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(54) Title: ASSAYS FOR GROWTH HORMONE SECRETAGOGUE RECEPTORS

(57) Abstract

An assay for the detection of growth hormone secretagogue receptors and growth hormone secretagogue related receptors is described. As these receptors are a member of the G protein coupled receptors, a subunit of the G protein must be present in order for expression to be detected. A similar assay is described where the presence of growth hormone secretagogues are detected.

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TITLE OF THE INVENTION

ASSAYS FOR GROWTH HORMONE SECRETAGOGUE RECEPTORS

FIELD OF THE INVENTION

5   This invention relates to an assay which involves identification of cell membrane receptors, specifically growth hormone secretagogoue receptors (GHSRs). By varying the protocol, receptor ligands can be identified, or the presence of a GHSR can be identified.

BACKGROUND OF THE INVENTION

10   Growth hormone (GH) is an anabolic hormone capable of promoting linear growth, weight gain and whole body nitrogen retention. Classically, GH is thought to be released primarily from the somatotroph cells of the anterior pituitary under the coordinate regulation of two hypothalamic hormones, growth hormone releasing

15 factor (GHRF or GRF) and somatostatin. Both GHRF stimulation and somatostatin inhibition of the release of GH occurs by the specific engagement of receptors on the cell membrane of the somatotroph.

          Recent evidence has been mounting which suggests that GH release is also stimulated by a group of short peptides termed the growth

20 hormone releasing peptides (GHRP; GHRP-6, GHRP-2 [hexarelin]) These peptides are described, for example, in U.S. Patent No. 4,411,890, PCT Patent Pub. No. WO 89/07110, PCT Patent Pub. No. WO 89/07111, PCT Patent Pub. No. WO 93/04081, and *J. Endocrinol*

25 *Invest.*, *15*(Suppl 4), 45 (1992). These peptides function by selectively bind to a distinct somatotroph cell membrane receptor, the growth hormone secretagogue receptor (GHSR). A medicinal chemical approach has resulted in the design of several classes of orally-active, low molecular weight, non-peptidyl compounds which bind specifically

30 to this receptor and result in the pulsatile release of GH. Such compounds possessing growth hormone secretagogue activity are disclosed, for example, in the following: U.S. Patent No. 3,239,345; U.S. Patent No. 4,036,979; U.S. Patent No. 4,411,890; U.S. Patent No. 5,206,235; U.S. Patent No. 5,283,241; U.S. Patent No. 5,284,841; U.S.

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Patent No. 5,310,737; U.S. Patent No. 5,317,017; U.S. Patent No. 5,374,721; U.S. Patent No. 5,430,144; U.S. Patent No. 5,434,261; U.S. Patent No. 5,438,136; U.S. Patent No. 5,494,919; U.S. Patent No. 5,494,920; U.S. Patent No. 5,492,916; EPO Patent Pub. No. 0,144,230;

5 EPO Patent Pub. No. 0,513,974; PCT Patent Pub. No. WO 94/07486; PCT Patent Pub. No. WO 94/08583; PCT Patent Pub. No. WO 94/11012; PCT Patent Pub. No. WO 94/13696; PCT Patent Pub. No. WO 94/19367; PCT Patent Pub. No. WO 95/03289; PCT Patent Pub. No. WO 95/03290; PCT Patent Pub. No. WO 95/09633; PCT Patent

10 Pub. No. WO 95/11029; PCT Patent Pub. No. WO 95/12598; PCT Patent Pub. No. WO 95/13069; PCT Patent Pub. No. WO 95/14666; PCT Patent Pub. No. WO 95/16675; PCT Patent Pub. No. WO 95/16692; PCT Patent Pub. No. WO 95/17422; PCT Patent Pub. No. WO 95/17423; PCT Patent Pub. No. WO 95/34311; PCT Patent Pub.

15 No. WO 96/02530; *Science, 260*, 1640-1643 (June 11, 1993); *Ann. Rep. Med. Chem., 28*, 177-186 (1993); *Bioorg. Med. Chem. Ltrs., 4*(22), 2709-2714 (1994); and *Proc. Natl. Acad. Sci. USA 92*, 7001-7005 (July 1995).

20 The use of such orally-active agents which stimulate the pulsatile release of GH would be a significant advance in the treatment of growth hormone deficiency in children and adults as well as provide substantial benefit under circumstances where the anabolic effects of GH might be exploited clinically (e.g. post-hip fracture rehabilitation, the

25 frail elderly and in post-operative recovery patients).

Cell membrane receptors which are of low abundance on the cells can be difficult to isolate, clone and characterize. In the past, assays to identify a receptor in a mammalian cell or frog oocyte generally have depended on either: 1) directly detecting a receptor-

30 ligand interaction, such as by binding of a radiolabeled ligand; or 2) indirectly detecting receptor-ligand binding by detecting either an intracellular event (such as calcium mobilization, or the identification of, for instance a calcium activated current) or an extracellular event (such as hormone secretion), that is the consequence of the ligand

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binding to its receptor. Most cloned receptors, which have been isolated using a functional expression assay have relied on immortalized cell lines or tumor derived tissues which are enriched for the receptor of interest.

5   There are numerous receptors which cannot be readily identified using these types of assays, due to: 1) a paucity of biochemical information about the protein; 2) the low abundance of receptors present on the cell; and/or 3) the lack of a cell line or tumor material expressing the receptor. It would be desirable to develop an assay

10   which can be used to identify and characterize cell receptors not amenable to study by conventional means.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to an assay method to determine the

15   presence of a nucleic acid which encodes a G protein-linked cell membrane receptor comprising: a) introducing at least one nucleic acid suspected of encoding a G protein cell membrane receptor into a cell;

        b)   introducing a G-protein subunit into the cell;
        c)   introducing a detector molecule or a nucleic acid

20   encoding a detector molecule into the cell, wherein
                the detector molecule responds directly or indirectly
                to a G-protein receptor-ligand binding event;
        d)   contacting the cell with a receptor ligand; and
        e)   determining whether the oligonucleotide encoded a

25   receptor by monitoring the detector molecule.

In one preferred embodiment the cell does not naturally express the receptor on its cell membrane. In other preferred embodiments of the assay, the receptor is a member of the growth

30   hormone secretagogue family of receptors, such as a growth hormone secretagogue receptor (GHSR) or a growth hormone secretagogue related receptor (GHSRR). Thus, another aspect of this invention is an assay method to determine the presence of a nucleic acid which encodes

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a member of the growth hormone secretagogue receptor family comprising:

a)   introducing at least one nucleic acid suspected of encoding a GHSR or GHSRR into a cell which does not naturally express the receptor on its cell membrane;

b)   introducing a G-protein subunit into the cell;

c)   introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand or GHSRR-ligand binding event;

d)   contacting the cell with a growth hormone secretagogue; and

e)   determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

A further embodiment of this invention is an assay to determine the presence of a growth hormone secretagogue. Thus, this invention also comprises a method to determine the presence of a growth hormone secretagogue comprising:

a)   introducing a nucleic acid which encodes a growth hormone secretagogue receptor into a cell under conditions so that growth hormone secretagogue receptor is expressed;

b)   introducing a G-protein subunit into the cell;

c)   introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand binding event;

d)   contacting the cell with a compound suspected of being a growth hormone secretagogue; and

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e)   determining whether the compound is a growth
hormone secretagogue by monitoring the detector
molecule.

5   BRIEF DESCRIPTION OF THE FIGURES
FIGURE 1 is the DNA of Swine GHSR (Type I) contained
in Clone 7-3.
FIGURE 2 is the amino acid sequence of swine GHSR
encoded by the DNA of Figure 1.
10   FIGURE 3 is the entire open reading frame of the Type I
clone, of Figure 1.
FIGURE 4 is the DNA of Swine GHSR (Type II) contained
in Clone 1375.
FIGURE 5 is the amino acid sequence of swine GHSR
15   (Type II) encoded by the DNA of Figure 4.
FIGURE 6 is the DNA for human GHSR (Type I) contained
in Clone 1146.
FIGURE 7 is the amino acid sequence of human GHSR
(Type I) encoded by the DNA of Figure 6.
20   FIGURE 8 is the entire open reading frame of Type I
GHSR, encoded by DNA sequence of Figure 6.
FIGURE 9 is the DNA for human GHSR (Type II)
contained in Clone 1141.
FIGURE 10 is the amino acid sequence of human GHSR
25   (Type II) encoded by Clone 1141.
FIGURE 11 is the DNA for human GHSR (Type I)
contained in Clone 1143.
FIGURE 12 is the amino acid sequence of human GHSR
(Type I) encoded by Clone 1143.
30   FIGURE 13 compares the ORF of swine Type I (lacking
the MET initiator of the full length GHSR and lacking 12 additional
amino acids) to the homologous domain of swine Type II receptors.

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FIGURE 14 compares the homologous domain of human Type I and Type II receptors (the amino terminal sequence lacks the MET initiator and four additional amino acids).

FIGURE 15 compares the ORFs of swine Type I and human Type I receptors (the amino terminal sequence lacks the MET initiator and 12 additional amino acids).

FIGURE 16 compares full length swine Type II and human Type II receptors.

FIGURE 17 is a schematic diagram depicting the physical map of swine and human growth hormone secretagogue receptor cDNA clones.

FIGURE 18 is a graph demonstrating the pharmacology of the expressed swine and human growth hormone secretagogue receptors in *Xenopus* oocytes using the aequorin bioluminescence assay.

FIGURE 19 is a table demonstrating the pharmacology of the expressed swine and human growth hormone secretagogue receptors in *Xenopus* oocytes using the aequorin bioluminescence assay and various secretagogues.

FIGURE 20 is a graph representing the pharmacology of the pure expressed swine growth hormone secretagogue receptor in COS-7 cells using the ^{35}S-labeled Compound A binding assay.

FIGURE 21 is a table representing the competition analysis with the pure expressed swine growth hormone secretagogue receptor in COS-7 cells using the ^{35}S-labeled Compound A binding assay and various secretagogues and other G-protein coupled- receptors (GPC-receptors) ligands in a competition assay.

FIGURE 22 is the amino acid sequence of the full length human GHSR (Type I) encoded by clone 11304.

FIGURE 23A and 23B are graphs of measurement of [^{35}S]-Compound A binding to swine anterior pituitary membranes. 23A shows results of saturation experiments using a fixed amount of membrane. 23B shows saturation isotherms analyzed by Scatchard analysis.

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FIGURE 24 shows the inhibition of [^{35}S]-Compound A binding to porcine anterior pituitary membranes by various compounds.

FIGURE 25 shows the effect of GHRP-6 on specific [^{35}S]-Compound A binding to porcine anterior pituitary membranes at equilibrium.

FIGURE 26 shows the effects of GTP-γ-S and nucleotide on the specific [^{35}S]-Compound A binding to porcine anterior pituitary membranes.

FIGURE 27 is the rat GHSR DNA sequence from the Met Initiation codon to the Stop codon. This sequence includes an intron.

FIGURE 28 is the open reading frame only of the rat GHSR of Figure 27.

FIGURE 29 is the deduced amino acid sequence of the ORF of Figure 28.

FIGURE 30 shows expression of functional rat GHSR in transfected HEK-293 cells.

As used throughout the specification and claims, the following definitions apply:

"Ligands" are any molecule which binds to a GHSR of this invention. Ligands can have either agonist, partial agonist, partial antagonist or antagonist activity.

"Growth hormone secretagogue" or "GHS" is any compound or agent that directly or indirectly stimulates or increases the release of growth hormone in an animal.

"Compound A" is (N-[1(R)-[1,2-dihydro-1-methane-sulfonlylspiro[3H-indole-3,4'-piperidin]-1'-yl)carbonyl]-2-(phenyl-methyloxy)-ethyl]-2-amino-2-methyl-propanamide, described in Patchett et al. 1995 Proc. Natl. Acad. Sci 92: 7001-7005.

"Compound B" is (3-amino-3-methyl-N-(2,3,4.5-tetrahydro-2-oxo-1{2'-(1H-tetrazol-5-yl)(1,1'-biphenyl)-4-yl]-methyl}1H-1-benzazepin-3(R)yl-butanamide, described in Patchett et al. 1995 Proc. Natl. Acad. Sci. 92: 7001-7005.

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This invention relates to assays for members of the growth
hormone secretagogue receptor family of proteins, which includes
growth hormone secretagogue receptors and growth hormone
secretagogue related receptors. The growth hormone secretagogue
5   receptor proteins, growth hormone receptor related proteins, nucleic
acids encoding them and methods of making them using genetic
engineering techniques are the subject of co-pending United States
Provisional Patent Application Nos. 60/008,582, filed December 13,
1995 and (Attorney Docket No. 19589PV2), filed herewith.
1 0

The proteins of this invention were found to have structural
features which are typical of the 7-transmembrane domain (TM)
containing G-protein linked receptor superfamily (GPC-R's or 7-TM
receptors) receptors. Thus growth hormone secretagogue receptors
1 5   make up new members of the GPC-R family of receptors. The intact
receptors of this invention were found to have the general features of
GPC-R's, including seven transmembrane regions, three intra- and
extracellular loops, and the GPC-R protein signature sequence. The
transmembrane domains and the GPC-receptor signature sequence are
2 0   noted in the protein sequences of the Type 1 GHS receptor in Figures 3
and 8. Not all regions are required for functioning.

The GHSRs share some sequence homology with previously
cloned GPC-receptors including the rat and human neurotensin receptor
(approximately 32% identity) and the rat and human TRH receptor
2 5   (approximately 30% identity).

The GHSRs were isolated and characterized using
expression cloning techniques in *Xenopus* oocytes. The cloning was
made difficult by three factors. First, prior to this invention, there was
very little information available about both the biochemical
3 0   characteristics, and the intracellular signaling/effector pathways of the
proteins. Thus, cloning approaches which depend on the use of protein
sequence information for the design of degenerate oligonucleotides to
screen cDNA libraries or utilize the PCR could not be effectively
utilized. Therefore, receptor bioactivity needed to be determined.

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Secondly, the growth hormone secretagogue receptor does not occur in abundance-- it is present on the cell membrane in about 10 fold less concentration than most other membrane receptors. In order to successfully clone the receptors, exhaustive precautions had be taken to ensure that the GHSR was represented in a cDNA library to be screened. This required: 1) isolation of intact, undegraded and pure poly (A)+ mRNA; 2) optimization of cDNA synthesis to maximize the production of full-length molecules; and 3) a library of larger size than normal needed to be screened (approximately 0.5 to 1 x 10^7 clones) to increase the probability that a functional cDNA clone may be obtained.

Thirdly, no permanent cell line which expresses these receptors is known. Therefore, primary pituitary tissue had to be used as a source for mRNA or protein. This is an added difficulty because most primary tissues express lower amounts of a given receptor than an immortalized cell line or tumor tissues. Further, the surgical removal of a pig pituitary and extraction of biologically active intact mRNA for the construction of a cDNA expression library is considerably more difficult than the extraction of mRNA from a tissue culture cell line. Along with the need to obtain fresh tissue continuously, there are problems associated with its intrinsic inter-animal and inter-preparation variability.

One aspect of this invention is directed to the development of an extremely sensitive, robust, reliable and high-throughput screening assay which could be used to identify portions of a cDNA library encoding the receptor.

The ability to identify cDNAs which encode growth hormone secretagogue receptors depended upon two discoveries made in accordance with this invention: 1) that growth hormone secretagogue receptor-ligand binding events are transduced through G proteins; and 2) that a particular G protein subunit, such as $G\alpha 11$, must be present in the cells in order to detect receptor activity. Only when these two discoveries were made could an assay be devised to detect the presence of GHSR encoding DNA sequences.

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Determination that GHSR is distinct from the Growth Hormone
Receptor

     A radioreceptor assay using high specific activity (700-
1,100 Ci/mmole) [^{35}S]-labeled Compound A (a known GHS) as ligand
5   was developed. Saturable, high affinity binding was detected in porcine
anterior pituitary membranes (FIGURE 23A). Scatchard analysis
(FIGURE 23B) indicated the presence of a single class of high affinity
sites with an apparent dissociation constant (K$_D$) of 161±11 pM and a
concentration (B$_{max}$) of 6.3±0.6 fmol/mg of protein (n=4). A similar
10   specific high affinity binding was detected in rat pituitary membranes
indicating a K$_D$ value of 180±9 pM and B$_{max}$ of 2.3±1.1 fmol/mg
protein (n=3).
     The high affinity binding to the GHSR makes up yet
another aspect of this invention. This invention is also directed to a
15   method of identifying novel GHSR proteins comprising labeling a
known ligand, exposing it to a putative GHSR protein and determining if
binding occurs.
     The specificity of [^{35}S]-Compound A binding was
established by determining the ability of GH secretagogues to compete
20   with the radioligand for the binding sites (FIGURE 24). Unlabeled
Compound A completely displaced [^{35}S]-Compound A from specific
binding sites with an inhibition constant, K$_i$, of 240 pM which is similar
to the K$_D$ value determined by Scatchard analysis. Other GHSs, GHRP-
6 (K$_i$ 6.3 nM), and peptide antagonist Compound B (K$_i$ 63 nM) had
25   affinities of 3.8, 0.6 and 0.4%, respectively, of that of Compound A.
Compound C, the biologically inactive stereoisomer of Compound B,
competed poorly with [^{35}S]-Compound A binding. The saturation
isotherm for [^{35}S]-Compound A binding analyzed by double reciprocal
plot showed that GHRP-6 inhibition was overcome by increasing
30   concentration of [^{35}S]-Compound A (FIGURE 25). This result shows
that GHRP-6 interacts competitively with Compound A in the same
binding site. Similarly, Compound B was shown to be a competitor of
[^{35}S]-Compound A binding. The most potent agonists had the highest
affinities for pituitary receptor sites. Compounds which did not

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compete with [^{35}S]-Compound A at 1 μM included GHRH, somatostatin, met-enkephalin, substance P, galanin, gonadotropin releasing hormone, thyrotropin releasing hormone, gastrin releasing peptide, PHM-27, melanocyte stimulating hormone, pituitary adenylate cyclase activating
5   polypeptide-38, phenoxybenzamine, dopamine, bromocriptine, methoxamine, benoxathian, isoproterenol, propanolol and clonidine.

      A GHSRR gene may be identified by hybridizing a cDNA encoding a GHSR to a genomic DNA, under relaxed post-hybridizational washing conditions (6 X SSC at 30°C) or moderate post-
10 hybridizational washing conditions (6 X SSC at 45°C). The hybridized area can be identified, isolated and the GHSRR can be cloned and the receptor expressed using conventional techniques.

Determination that GHSR is a G-Protein Receptor
15   To study whether the [^{35}S]-Compound A specific binding site was G-protein linked, the effects of stable GTP analogs GTP-γ-S and GMP-PNP on [^{35}S]-Compound A binding were studied. GTP-γ-S and GMP-PNP were found to be potent inhibitors of [^{35}S]-Compound A binding with IC$_{50}$ values of 30 and 110 nM, respectively (FIGURE 26).
20 ATP-γ-S was ineffective. In addition, in the absence of Mg^{2+}, only 15-25% of specific binding of [^{35}S]-Compound A binding was detected in comparison with control (10 mM Mg^{2+}) suggesting that the specific binding of [^{35}S]-Compound A required the presence of Mg^{2+} regulate GH release *in vivo*) do not bind to the Compound A site. From these
25 data, one can conclude that the receptor is G-protein linked.

      When the GHSR is bound by ligand (a growth hormone secretagogue), the G-proteins present in the cell activate phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme which releases intracellular signaling molecules (diacylglycerol and
30 inositol tri-phosphate), which in turn start a cascade of biochemical events that promote calcium mobilization. In accordance with this invention, detection of this biochemical cascade can be used as the basis of an assay.

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Virtually any convenient eukaryotic cell may be used in the assay of this invention. These would include oocytes (preferred ones are from *Xenopus sp.*) but cell lines may be used as well as Examples of preferred cell lines are mammalian cell lines, including COS, HEK-293,

5   CHO, HeLa, NS/O, CV-1, GC, GH3 and VERO.

One important component of the assay is a detector molecule. Preferably, the detector molecule is responsive to an intracellular event which is part of the biochemical cascade initiated by GHS-GHSR binding. One class of preferred detector molecules can

10   respond to changes in calcium concentrations. A preferred detector molecule which responds to calcium concentrations is aequorin (a jellyfish photoprotein) which acts on the substrate coelenterazine. Other detector molecules include calcium chelators with fluorescence capabilities, such as FURA-2 and indo-1.

15   The detector molecule itself may be introduced into the cell, or nucleotides which encode the detector molecule may be introduced into the cell, under conditions which will allow the expression of the detector molecule. Generally, it is preferred to introduce nucleotides, such as DNA which encode the detector molecule

20   into the cell, under conditions wherein the cell will express the detector molecule.

Heterotrimeric G proteins, consisting of α, β and γ subunits, serve to relay information from cell surface receptors to intracellular effectors, such as phospholipase C and adenylate cyclase.

25   The G-protein alpha subunit is an essential component of the intracellular signal transduction pathway activated by receptor-ligand interaction. In the process of ligand-induced GPCR activation, the Gα subunit of a trimeric G$\alpha\beta\gamma$ complex will exchange its bound GDP for GTP and dissociate from the $\beta\gamma$ heterodimer. The dissociated Gα-

30   protein serves as the active signal transducer, often in concert with the $\beta\gamma$ complex, thus starting the activation of the intracellular signal transduction pathway. G-alpha subunits are classified into sub-families based on sequence identity and the main type of effectors are coupled:

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G_s, activate adenylate cyclase, $G_i/o/t$, inhibit adenylate cyclase, $G_q/11$, activate PI-PLC, and $G_{12}/13$, effector unknown.

The expression of several receptors in heterologous cells has been shown to be increased by the co-expression of certain $G\alpha$

5 subunits. This observation formed the basis for the rationale to use $G\alpha$ subunits of several sub-families in conjunction with a source of GHSR (swine poly A^+ mRNA) to test if a GHS-induced functional response could be measured in the *Xenopus* oocyte system. GHS-induced responses were detected and were found to be strictly dependent on

10 $G\alpha_{11}$ co-expression, a unprecedented finding outlining the specificity of the interaction. The finding that the expression of the GPCR could be fully dependent on the addition of a single G-protein subunit was unexpected, since in all previously published work the addition of a G-protein subunit modulated an already existing activity. Here a

15 previously absent signal was fully restored. This finding indicated that the lack of a signal in *Xenopus* eggs was fully dependent on a G-protein subunit as the limiting factor.

In conducting the assay, either the subunit itself or a nucleic acid encoding the subunit, or both may be added, and the addition events

20 need not occur together.

Next, a nucleic acid or pool of nucleic acids, wherein at least one nucleic acid is suspected of encoding a GHSR or GHSRR is introduced into the cell. When trying to identify a possible GHSR or GHSRR gene from a large library, it is often more efficient to use a

25 pool of nucleic acids, each nucleic acid being different from the other nucleic acids in the pool.

After the nucleic acid(s) suspected of encoding a GHSR or GHSRR is introduced into the cell, the cell is exposed to a known growth hormone secretagogue, such as Compound A (L-163,191). Any

30 other growth hormone secretagogue may also be used. Preferred ones include: N-[1(R)-[(1,2-dihydro-1-methanesulfonylspiro[3H-indole-3,4'-piperidin]-1'-yl)carbonyl]-2-(phenylmethyloxy)ethyl]-2-amino-2-methylpropanamide, or 3-amino-3-methyl-N-(2,3,4,5-tetrahydro-2-oxo-1-[[2'-1H-tetrazol-5-yl)(1,1'-biphenyl)-4-yl]methyl]-1H-1-benzazepin-

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3(R)-yl-butanamide, or a compound disclosed, for example, in the
following: U.S. Patent No. 3,239,345; U.S. Patent No. 4,036,979; U.S.
Patent No. 4,411,890; U.S. Patent No. 5,206,235; U.S. Patent No.
5,283,241; U.S. Patent No. 5,284,841; U.S. Patent No. 5,310,737; U.S.
5   Patent No. 5,317,017; U.S. Patent No. 5,374,721; U.S. Patent No.
5,430,144; U.S. Patent No. 5,434,261; U.S. Patent No. 5,438,136; U.S.
Patent No. 5,494,919; U.S. Patent No. 5,494,920; U.S. Patent No.
5,492,916; EPO Patent Pub. No. 0,144,230; EPO Patent Pub. No.
0,513,974; PCT Patent Pub. No. WO 94/07486; PCT Patent Pub. No.
10   WO 94/08583; PCT Patent Pub. No. WO 94/11012; PCT Patent Pub.
No. WO 94/13696; PCT Patent Pub. No. WO 94/19367; PCT Patent
Pub. No. WO 95/03289; PCT Patent Pub. No. WO 95/03290; PCT
Patent Pub. No. WO 95/09633; PCT Patent Pub. No. WO 95/11029;
PCT Patent Pub. No. WO 95/12598; PCT Patent Pub. No. WO
15   95/13069; PCT Patent Pub. No. WO 95/14666; PCT Patent Pub. No.
WO 95/16675; PCT Patent Pub. No. WO 95/16692; PCT Patent Pub.
No. WO 95/17422; PCT Patent Pub. No. WO 95/17423; PCT Patent
Pub. No. WO 95/34311; PCT Patent Pub. No. WO 96/02530; *Science*,
260, 1640-1643 (June 11, 1993); *Ann. Rep. Med. Chem.*, *28*, 177-186
20   (1993); *Bioorg. Med. Chem. Ltrs.*, *4*(22), 2709-2714 (1994); and *Proc.
Natl. Acad. Sci. USA 92*, 7001-7005 (July 1995), or any other growth
hormone secretagogue.

If one or more of the nucleic acids does encode a GHSR, or
25   GHSRR, then the secretagogue ligand will bind the receptor, G-protein
will be activated, the calcium level will fluctuate, and the detector
molecule will change so that it can be monitored. For the system using
aequorin and coelenterazine, receptor-GHS binding will produce
measurable bioluminescence.
30   If the procedure used a complex pool of nucleic acids, one
or more of which may encode the receptor, then further screening will
be necessary to determine which nucleic acid is responsible for encoding
GHSR or GHSRR. Once a positive result is found, the procedure can be
repeated with a sub-division of the nucleic acid pool (for example,

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starting with approximately 10,000 nucleic acids, then using approximately 1,000, then approximately 500, then approximately 50, and then pure). In this procedure, RNA pools are preferred.

Using this general protocol in *Xenopus* oocytes with a

5   swine cDNA expression library, Clone 7-3 was identified as containing nucleic acid encoding a swine growth hormone secretagogue receptor. The clone is approximately 1.5 kb in size, and downstream from the presumed initiator methionine (MET), contains an open reading frame (ORF) encoding 302 amino acids (M_r= 34,516). The DNA and deduced

10 amino acid sequence is given in FIGURES 1 and 2. When hydropathy analysis (e.g. Kyte-Doolittle; Eisenberg, Schwartz, Komaron and Wall) is performed on the protein sequence of clone 7-3, only 6 predicted transmembrane domains are present downstream of the presumed MET initiator. However, translation of the longest ORF encoded in clone 7-3

15 encodes a protein of 353 amino acids (M_r= 39,787), but is devoid of an apparent initiator MET (FIGURE 3). Seven transmembrane segments are encoded in the longer, 353 amino acid protein in which a MET translation initiation codon located upstream of TM1 is absent (FIGURE 3). Thus, clone 7-3 appears truncated at its amino terminus, but is fully

20 functional, demonstrating that clone 7-3 is a functional equivalent of a native GHSR.

The resultant cDNA clone (or shorter portions of for instance only 15 nucleotides long) may be used to probe libraries under hybridization conditions to find other receptors which are similar

25 enough so that the nucleic acids can hybridize, and is particularly useful for screening libraries from other species. Using this procedure, additional human, swine and rat GHSR cDNAs have been cloned and their nucleotide sequence determined. In this step, one of ordinary skill in the art will appreciate that the hybridization conditions can vary from

30 very stringent to relaxed. Proper temperature, salt concentrations, and buffers are well known. As used herein, "standard post hybridizational washing" conditions mean 6 x SSC at 55°C. "Relaxed post hybridizational washing" conditions means 6 x SSC at 30°C.

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A swine pituitary library, a human pituitary library, and a rat pituitary library were hybridized with a radiolabeled cDNA derived from the open reading frame of the swine GHSR clone 7-3. Twenty one positive human GHSR cDNA clones were isolated and five swine library pools yielded a strong hybridization signal and contained clones with inserts larger than clone 7-3, as judged from their insert size on Southern blots. A single rat cDNA clone was also isolated.

Nucleotide sequence analysis revealed two types of cDNAs for both the human and swine GHSR cDNAs. The first (Type I) encodes a protein represented by clone 7-3, encoding 7-TM domains (the amino acid sequence of a full length human clone 11304 is shown in FIGURE 22). The full length open reading frame extends 13 amino acids beyond the largest predicted open reading frame of clone 7-3, (353 amino acids).

The second (type II) diverges in its nucleotide sequence from the type I cDNA at its 3'-end, at the second predicted amino acid of TM-6. In the type II cDNAs, TM-6 is truncated and fused to a short contiguous reading frame of only 24 amino acids, followed by a translation stop codon. Swine clone 1375 is an example of a Type II cDNA (FIGURES 4 and 5). These 24 amino acids beyond TM-6 are highly conserved when compared between human and swine cDNAs. The DNA and amino acid sequences of the human GHSR Type I and II are given in FIGURES 6-12 and 22. A predicted full length cDNA encoding the human Type I receptor, that is, a molecule encoding 7-TM domains with an initiator MET in a favorable context preceded by an inframe termination codon is isolated, and termed clone 11304. The predicted ORF of clone 11304 for the full length Type I GHSR measures 366 amino acids (M_r= 41,198; FIGURE 22). A full length human Type II cDNA encodes a polypeptide of 289 amino acids (M_r=32,156; FIGURES 9 and 10). Sequence alignments performed at both the nucleic acid and protein levels show that Type I and II GHSR's are highly related to each other and across species (FIGURES 13-16). The human and swine GHSR sequences are 93% identical and 98% similar at the amino acid level.

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The nucleotide sequence encoding the missing amino terminal extension of swine Type I clone 7-3 is derived from the full length human Type I clone as well as the human and swine Type II cDNAs. The reading frame of the full length clones extended 13 amino
5   acids beyond the amino terminal sequence of clone 7-3 and this sequence was conserved in 12/13 amino acid residues when compared between human and swine. The amino terminal extension includes a translation initiator methionine in a favorable context according to Kosak's rule, with the reading frame further upstream being interrupted by a stop
10   codon. A schematic physical map of Type I and II swine and human cDNA clones is given in FIGURE 17.

The rat clone was also further investigated. Sequence analysis revealed the presence of a non-coding intronic sequence at nt 790 corresponding to a splice-donor site (see FIGURES 27, 28, and 29.)
15   The G/GT splice-donor site occurs two amino acids after the completion of the predicted transmembrane domain 5 (leucine 263), thus dividing the rat GHSR into an amino-terminal segment (containing the extra cellular domain, TM-1 through TM-5, and the first two intra- and extra- cellular loops) and a carboxy-terminal segment (containing TM-
20   6, TM-7, the third intra- and extra- cellular loops, and the intra-cellular domain). The point of insertion and flanking DNA sequences are highly conserved, and also present in both human and swine Type I and II cDNAs.

Comparison of the complete open reading frame encoding
25   the rat GHSR protein to human and swine homologs reveals a high degree of sequence identity (rat vs. human, 95.1%; rat vs. swine 93.4%).

Human and swine Type I cRNAs expressed in oocytes were functional and responded to concentrations Compound A ranging from
30   1 μM to as low as 0.1 nM in the aequorin bioluminescence assay. Human or swine Type II-derived cRNAs that are truncated in TM-6 failed to give a response when injected into oocytes and these represent a receptor subtype which may bind the GHS, but cannot effectively activate the intracellular signal transduction pathway. In addition the

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Type II receptor may interact with other proteins and thus reconstitute a functional GHSR. Proteins such as these which may have ligand-binding activity, but are not active in signal transduction are particularly useful for ligand-binding assays. In these cases, one may also over-express a
5   mutant protein on the cell membrane and test the binding abilities of putative labeled ligands. By using a non-signaling mutant which is constitutively in a high affinity state, binding can be measured, but no adverse metabolic consequences would result. Thus use of non-signaling mutants are an important aspect of this invention.

10   The pharmacological characterization of human Type I, swine Type I and rat receptors in the aequorin bioluminescence assay in oocytes is summarized in FIGURES 18, 19 and 30. Peptidyl and non-peptidyl bioactive GHS's were active in a similar rank order of potency as observed for the native pituitary receptor. Independent confirmatory
15   evidence that the Type I GHSR (shown for swine clone 7-3) encodes a fully-functional GHSR is given by the finding that when clone 7-3 is expressed transiently in mammalian COS-7 cells, high affinity (KD ~ 0.2 nM), saturable (B_{max}~80 fmol/mg protein) and specific binding (> 90 % displaced by 50 nM unlabeled Compound A) is observed for ^{35}S-
20   Compound A (FIGURES 20-21).

By varying the parameters of the above assays, one can search for other unknowns. For example, in the assay which detects whether a nucleic acid which encodes a GHSR or GHSRR is present, one can modify the assay so that it detects whether a GHS is present. In this
25   embodiment, a nucleic acid encoding GHSR or GHSRR is introduced into the cell, as well as a nucleic acid encoding a detector molecule, and a G protein subunit. The cell is contacted with at least one compound which is a putative GHS. If the compound is a GHS, then the GHS will bind the GHSR or GHSRR, and the resultant intracellular events can be
30   detected by monitoring the detector molecule. If the compound is not a GHS, then no such activity will be detected. This GHS assay forms yet another aspect of this invention.

A further aspect of this invention are novel ligands which are identified using the above assay.

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Expression of several receptors in heterologous cells has been shown to be increased by the co-expression of certain Gα subunits. This observation formed the basis for the rationale to the use of Gα subunits of several sub-families in conjunction with a source of GHSR (swine poly[A+] mRNA) to test if a GHS-induced functional response could be measured in the *Xenopus* oocyte system. GHS-induced responses were detected and were found to be strictly dependent on Gα11 co-expression, an unprecedented finding outlining the specificity of the interaction. Thus another aspect of this invention is a method of detecting a GHS response comprising co-expressing a Gα11 protein subunit in a cell also expressing a GHSR, exposing the cell to a GHS, and detecting the response.

The presence of Gα11 was essential in using poly A+ RNA or complex cRNA pools (i.e. 10.000 cRNAs). However, once a pure clone was obtained the requirement for the G-protein addition was no longer essential. This indicates that the need for G-protein addition depended on the purity of the nucleic acid, the most sensitive assay requiring Gα subunit addition. Thus another aspect of this invention is a method of determining the presence of an nucleic acid which encodes a growth hormone secretagogue receptor or growth hormone secretagogue related receptor comprising:

a)   introducing a nucleic acid suspected of encoding a GHSR or GHSRR into a cell which does not naturally express the receptor on its cell membrane;

b)   introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a receptor-ligand binding event;

c)   contacting the cell with a growth hormone secretagogue; and

d)   determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

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Similarly, another aspect of this invention is an assay
method to determine the presence of a growth hormone secretagogue
comprising:

a) introducing a nucleic acid which encodes a growth
hormone secretagogue receptor into a cell under
conditions so that growth hormone secretagogue
receptor is expressed;

b) introducing a detector molecule or a nucleic acid
encoding a detector molecule into the cell, wherein
the detector molecule is directly or indirectly
responsive to a GHSR-ligand binding event;

c) contacting the cell with a compound suspected of
being a growth hormone secretagogue; and

d) determining whether the compound is a growth
hormone secretagogue by monitoring the detector
molecule.

Ligands detected using assays described herein may be used
in the treatment of conditions which occur when there is a shortage of
growth hormone, such as observed in growth hormone deficient
children, elderly patients with musculoskeletal impairment and
recovering from hip fracture, patients with neurodegenerative diseases,
and patients recovering from coronary by-pass surgery, and
osteoporosis.

A GHS receptor, preferably imobilized on a solid support,
may be used diagnostically for the determination of the concentration of
growth hormone secretagogues, or metabolites thereof, in physiological
fluids, e.g., body fluids, including serum, and tissue extracts, as for
example in patients who are undergoing therapy with a growth hormone
secretagogue.

The administration of a GHS receptor to a patient may also
be employed for purposes of: amplifying the net effect of a growth
hormone secretagogue by providing increased downstream signal
following administration of the growth hormone secretagogue thereby

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diminishing the required dosage of growth hormone secretagogue; or
diminishing the effect of an overdosage of a growth hormone
secretagogue during therapy.

The following non-limiting Examples are presented to
better illustrate the invention.

EXAMPLE 1

Preparation of High Specific Activity Radioligand [^{35}S]-Compound A-

[^{35}S]-Compound A was prepared from an appropriate
precursor, N-[1(R)-[(1,2-dihydrospiro[3H-indole-3,4'-piperidin]-1'-yl)-
carbonyl]-2-(phenyl-methyloxy)ethyl]-2-amino-t-butoxycarbonyl-2-
methylpropanamide, using methane [^{35}S]sulfonyl chloride as described
in Dean DC, et al., 1995, In: Allen J, Voges R (eds) Synthesis and
Applications of Isotopically Labelled Compounds, John Wiley & Sons,
New York, pp. 795-801, Purification by semi-preparative HPLC
(Zorbax SB-phenyl column, 68% MeOH/water, 0.1% TFA, 5 ml/min)
was followed by N-t-BOC cleavage using 15% trifluroacetic acid in
dichloromethane (25°C, 3 hr) to give [methylsulfonyl-^{35}S]Compound A
in near quantitative yield. HPLC purification (Hamilton PRP-1 4.6x250
mm column, linear gradient of 50-75% methanol-water with 1 mM HCl
over 30 min, 1.3 ml/min) provided the ligand in >99% radiochemical
purity. The structure was established by HPLC coelution with unlabeled
Compound A and by mass spectral analysis. The latter method also
indicated a specific activity of ~1000 Ci/mmol.

EXAMPLE 2

Preparation of Pituitary Membranes

Frozen anterior pituitary glands from male swine (50-80
Kg) or from the Wistar male rats (150-200 g) were homogenized in a
tissue homogenizer in ice-cold buffer (50 mM Tris-HCl buffer, pH 7.4,
5 mM MgCl$_2$, 2.5 mM EDTA, 0.1% bovine serum albumin and 30
μg/ml bacitracin). The homogenates were centrifuged for 5 min at

●   ●

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1,400 xg and the resulting supernatants were then centrifuged at 34,000 xg for 20 min. The pellets were resuspended in same buffer to a 1,500 µg protein/ml and stored at -80°C. Protein was determined by a Bio-Rad method (Bio-Rad Laboratories, Richmond, CA).

5

EXAMPLE 3

Receptor Binding Assay

The standard binding solution contained: 400 m of 25 mM
10 Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, and 100 pM [^{35}S]-Compound A. Pituitary membranes (100 µl, 150 µg protein) were added to initiate the binding reaction. Aliquots were incubated at 20°C for 60 min and bound radioligand was separated from free by filtration through GF/C filters pretreated with 0.5% of polyethyl-
15 enimine in a Brandel cell harvester. The filters were washed three times with 3-ml of ice-cold buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA and 0.015% Triton X-100) and the radioactivity on the filters were counted in Aquasol 2. Specific binding was defined as the difference between total binding and nonspecific binding assayed
20 in 500 nM unlabeled Compound A. Specific bindings were 65-85 and 45-60% of total binding, in porcine and rat membranes, respectively. Assays were carried out in triplicate and experiments repeated at least three times.

25   ## EXAMPLE 4

Oocyte Preparation and Selection

Xenopus laevis oocytes were isolated and injected using standard methods previously described by Arena, et. al. 1991, *Mol.*
30 *Pharmacol.* 40, 368-374, which is hereby incorporated by reference. Adult female *Xenopus laevis* frogs (purchased from Xenopus One, Ann Arbor, MI) were anesthetized with 0.17% tricaine methanesulfonate and the ovaries were surgically removed and placed in a 60 mm culture dish (Falcon) containing OR-2 medium without calcium (82.5 mM NaCl, 2

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mM KCl, 2.5 mM sodium pyruvate, 1 mM $MgCl_2$, 100 μ/ml penicillin, 1 mg/ml streptomycin, 5 mM HEPES, pH=7.5; ND-96 from Specialty Media, NJ). Ovarian lobes were broken open, rinsed several times, and oocytes were released from their sacs by collagenase A digestion

5　(Boehringer-Mannheim; 0.2% for 2-3 hours at 18°C) in calcium-free OR-2. When approximately 50% of the follicular layers were removed, Stage V and VI oocytes were selected and placed in ND-86 with calcium (86 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 2.5 mM sodium pyruvate, 0.5 mM theopylline, 0.1 mM gentamycin, 5 mM

10　HEPES [pH=7.5]). For each round of injection, typically 3-5 frogs were pre-tested for their ability to express a control G-protein linked receptor (human gonadotropin-releasing hormone receptor) and show a robust phospholipase C intracellular signaling pathway (incubation with 1% chicken serum which promotes calcium mobilization by activation

15　of phospholipase C). Based on these results, 1-2 frogs were chosen for library pool injection (50 nl of cRNA at a concentration of 25 ng (complex pools) to 0.5 ng (pure clone) per oocyte usually 24 to 48 hours following oocyte isolation.

20　　　　　　　　　　　　　　　　EXAMPLE 5

mRNA Isolation

　　　　Total RNA from swine (50-80 kg, Yorkshire strain) pituitaries (snap-frozen in liquid nitrogen within 1-2 minutes of animal

25　sacrifice) was prepared by a modified phenol:guanidinium thiocyanate procedure (Chomczynski, et al, 1987 Anal. Biochem. 162, 156-159, which is hereby incorporated by reference), using the TRI-Reagent LS as per the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). Typically, 5 mg of total RNA was obtained from 3.5 g

30　wet weight of pituitary tissue. Poly (A)$^+$ RNA was isolated from total RNA by column chromatography (two passes) on oligo (dT) cellulose (Pharmacia, Piscataway, NJ). The yield of poly (A)$^+$ mRNA from total RNA was usually 0.5%. RNA from other tissues was isolated similarly.

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EXAMPLE 6

cDNA Library Construction

First-strand cDNA was synthesized from poly (A) $^+$ mRNA
using M-MLV RNAse (-) reverse transcriptase (Superscript, GIBCO-
BRL, Gaithersberg, MD) as per the manufacturer's instructions with an
oligo (dT)/Not I primer-adapter. Following second-strand cDNA
synthesis, double-stranded cDNA was subjected to the following steps:
1) ligation to EcoR I adapters, 2) Not I digestion, and 3) enrichment for
large cDNAs and removal of excess adapters by gel filtration
chromatography on a Sephacryl S-500 column (Pharmacia). Fractions
corresponding to high molecular weight cDNA were ligated to EcoR
I/Not I digested pSV-7, a eucaryotic expression vector capable of
expressing cloned cDNA in mammalian cells by transfection (driven by
SV-40 promoter) and in oocytes using *in vitro* transcripts (initiated
from the T7 RNA polymerase promoter). pSV-7 was constructed by
replacing the multiple cloning site in pSG-5 (Stratagene, La Jolla, CA;
Green, S. *et al*, 1988 *Nucleic Acids Res.* 16:369, which is hereby
incorporated by reference) with an expanded multiple cloning site.
Ligated vector:cDNA was transformed into *E.coli* strain DH10B
(GIBCO-BRL) by electroporation with a transformation efficiency of 1
x 10^6 pfu/10 ng double-stranded cDNA. The library contained
approximately 3 x 10^6 independent clones with greater than 95% having
inserts with an average size approximating 1.65 kb (range 0.8-2.8 kb).
Unamplified library stocks were frozen in glycerol at -70°C until
needed. Aliquots of the library were amplified once prior to screening
by a modification of a solid-state method (Kriegler, M. in *Gene
Transfer and Expression: A Laboratory Manual* Stockton Press, NY
1990). Library stocks were titered on LB plates and then the equivalent
of 500-1000 colonies was added to 13 ml of 2 x YT media containing
0.3% agarose and 100 μg/ml carbenicillin in a 14 ml round-bottom
polypropylene tube (Falcon). The bacterial suspension was chilled in a
wet ice bath for 1 hour to solidify the suspension, and then grown
upright at 37°C for 24 hrs. The resultant bacterial colonies were

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harvested by centrifugation at 2000 x g at RT for 10 min, resuspended in 3 ml 2X YT/ carbenicillin. Aliquots were taken for frozen stocks (5%) and plasmid DNA preparation.

5

EXAMPLE 7

Plasmid DNA Preparation and cRNA Transcription

Plasmid DNA was purified from pellets of solid-state grown bacteria (1000 pools of 500 independent clones each) using the

10 Wizard Miniprep kit according to the manufacturer's instructions (Promega Biotech, Madison, WI). The yield of plasmid DNA from a 14 ml solid-state amplification was 5-10 μg. In preparation for cRNA synthesis, 4 μg of DNA was digested with Not I, and the subsequent linearized DNA was made protein and RNase-free by proteinase K

15 treatment (10 μg for 1 hour at 37°C), followed by two phenol, two chloroform/isoamyl alcohol extractions, and two ethanol precipitations. The DNA was resuspended in approximately 15 μl of RNase-free water and stored at -70°C until needed. cRNA was synthesized using a kit from Promega Biotech with modifications. Each 50 μl reaction

20 contained: 5 μl of linearized plasmid (approximately 1 μg), 40 mM Tris-HCl (pH=7.5), 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.05 mg/ml bovine serum albumin, 2 units/ml RNasin, 800 μM each of ATP, CTP and UTP, 200 μM GTP, 800 μM m7G(5')ppp(5')G, 80 units of T7 RNA polymerase, and approximately

25 20,000 cpm of ^{32}P-CTP as a trace for quantitation of synthesized RNA by TCA precipitation. The reaction was incubated for 3 hrs. at 30°C; 20 units of RNase-free DNase was added, and the incubation was allowed to proceed for an additional 15 min. at 37°C. cRNA was purified by two phenol, chloroform/isoamyl alcohol extractions, two

30 ethanol precipitations, and resuspended at a concentration of 500 ng/ml in RNase-free water immediately before use.

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EXAMPLE 8

Aequorin Bioluminescence Assay (ABA) and Clone Identification

The ABA requires injection of library pool cRNA (25

5 ng/egg for pool sizes of 500 to 10,000) with aequorin cRNA (2 ng/egg) supplemented with the G-protein alpha subunit Gα11 (2 ng/egg). To facilitate stabilization of synthetic transcripts from aequorin and Gα11 plasmids, the expression vector pCDNA-3 was modified (termed pcDNA-3v2) by insertion (in the Apa I restriction enzyme site of the

10 polylinker) of a cassette to append a poly (A) tract on all cRNA's which initiate from the T7 RNA polymerase promoter. This cassette includes (5' to 3'): a Bgl II site, pA (20) and a Sfi I site which can be used for plasmid linearization. Polymerase chain reaction (PCR) was utilized to generate a DNA fragment corresponding to the open reading frame

15 (ORF) of the aequorin cDNA with an optimized Kosak translational initiation sequence (Inouye, S. et. al., 1985, Proc. Natl. Acad. Sci. USA 82:3154-3158). This DNA was ligated into pCDNA-3v2 linearized with EcoR I and Kpn I in the EcoR I/Kpn I site of pCDNA-3v2. Gα11 cDNA was excised as a Cla I/Not I fragment from the pCMV-5 vector

20 (Woon, C. et. al., 1989 J. Biol. Chem. 264: 5687-93), made blunt with Klenow DNA polymerase and inserted into the EcoR V site of pcDNA-3v2. cRNA was injected into oocytes using the motorized "Nanoject" injector (Drummond Sci. Co., Broomall, PA.) in a volume of 50 nl. Injection needles were pulled in a single step using a Flaming/Brown

25 micropipette puller, Model P-87 (Sutter Instrument Co) and the tips were broken using 53X magnification such that an acute angle was generated with the outside diameter of the needle being <3 μm. Following injection, oocytes were incubated in ND-96 medium, with gentle orbital shaking at 18°C in the dark. Oocytes were incubated for

30 24 to 48 hours (depending on the experiment and the time required for expression of the heterologous RNA) before "charging" the expressed aequorin with the essential chromophore coelenterazine. Oocytes were "charged" with coelenterazine by transferring them into 35 mm dishes containing 3 ml charging medium and incubating for 2-3 hours with

35 gentle orbital shaking in the dark at 18°C. The charging medium

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contained 10 μM coelenterazine (Molecular Probes, Inc., Eugene, OR.) and 30 μM reduced glutathione in OR-2 media (no calcium). Oocytes were then returned to ND-86 medium with calcium medium described above and incubation continued in the dark with orbital shaking until

5 bioluminescence measurements were initiated. Measurement of GHSR expression in oocytes was performed using a Berthold Luminometer LB953 (Wallac Inc., Gaithersburg, MD) connected to a PC running the Autolumat-PC Control software (Wallac Inc., Gaithersburg, MD). Oocytes (singly or in pairs) were transferred to plastic tubes (75 x 12

10 mm, Sarstedt) containing 2.9 ml Ca^{++}-free OR-2 medium. Each cRNA pool was tested using a minimum of 3 tubes containing oocytes. Bioluminescence measurements were triggered by the injection of 0.1 ml of 30 μM Compound A (1 μM final concentation) and recordings were followed for 2 min. to observe kinetic responses consistent with an

15 IP3-mediated response.

Pool S10-20 was prepared from the unfractionated swine pituitary cDNA library and was composed of 10 pools each of 1000 clones. S10-20 gave a positive signal on two luminometer instruments and the component pools were then individually tested for activity.

20 From the 10 pools of 1000 clones, only pool S271 gave a positive response. This pool was made from two pools of 500 clones designated P541 and P542. Again, only one of the pools, P541, gave a positive bioluminescent signal in the presence of 1 μM Compound A. At this point, the bacterial titer was determined in the glycerol stock of P541

25 such that dilutions could be plated onto LB agar plates containing 100 μg/ml carbenicillin to yield approximately 50 colonies per plate. A total of 1527 colonies were picked and replicated from 34 plates. The colonies on the original plates were then washed off, plasmids isolated, cRNA synthesized and injected into oocytes. cRNA prepared from 8 of

30 the 34 plates gave positive signals in oocytes. Two plates were selected and the individual colonies from these plates were grown up, plasmid isolated, cRNA prepared and injected into oocytes. A single clonal isolate from each plate (designated as clones 7-3 and 28-18) gave a

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positive bioluminescence response to 1 µM Compound A. Clone 7-3 was further characterized.

EXAMPLE 9

5

Receptor Characterization

DNA sequencing was performed on both strands using an automated Applied Biosystems instrument (ABI model 373) and manually by the dideoxy chain termination method using Sequenase II
10 (US Biochemical, Cleveland, OH). Database searches (Genbank 88, EMBL 42, Swiss-Prot 31, PIR 40, dEST, Prosite, dbGPCR), sequence alignments and analysis of the GHSR nucleotide and protein sequences were carried out using the GCG Sequence Analysis Software Package (Madison, WI; pileup, peptide structure and motif programs), FASTA
15 and BLAST search programs, and the PC/Gene software suite from Intelligenetics (San Francisco, CA; protein analysis programs). Northern blot analysis was conducted using total (20 µg/lane) or poly (A)+ mRNA (5-10 µg/lane) prepared as described above. RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde and
20 blotted to a nitrocellulose membrane. Blots were hybridized with a PCR generated probe encompassing the majority of the ORF predicted by clone 7-3 (nt 291 to 1132). The probe was radiolabeled by random-priming with $[\alpha]^{32}$P-dCTP to a specific activity of greater than 10^9 dpm/µg. Blots were pre-hybridized at 42°C for 4 hrs. in 5 X SSC, 5 X
25 Denhardt's solution, 250 µg/ml tRNA, 1% glycine, 0.075% SDS, 50 mM NaPO4 (pH 6) and 50% formamide. Hybridizations were carried out at 42°C for 20 hrs. in 5 X SSC, 1 X Denhardt's solution, 0.1% SDS, 50 mM NaPO4, and 50% formamide. RNA blots were washed in 2 X SSC, 0.2% SDS at 42°C and at -70°C. RNA size markers were 28S and
30 18S rRNA and in vitro transcribed RNA markers (Novagen). Nylon membranes containing EcoR I and Hind III digested genomic DNA from several species (Clontech; 10 mg/lane) were hybridized for 24 hrs. at 30°C in 6 X SSPE, 10 X Denhardt's, 1% SDS, and 50% formamide. Genomic blots were washed twice with room temperature 6 X SSPE,
35 twice with 55°C 6 X SSPE, and twice with 55°C 4 X SSPE. Additional

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swine GHSR clones from the swine cDNA library (described above) were identified by hybridization to plasmid DNA (in pools of 500 clones each) immobilized to nylon membranes in a slot-blot apparatus (Scheicher and Schuell). Pure clonal isolates were subsequently

5   identified by colony hybridization. Swine GHSR clones that extend further in a 5' direction were identified using 5' RACE procedures (Frohman, M. A., 1993 *Methods Enzymol.* 218:340-358, which is incorporated by reference) using swine pituitary poly $(A)^+$ mRNA as template.

10

EXAMPLE 10

Human GHSR

Human pituitary homologues of the swine GHSR were

15   obtained by screening a commercially available cDNA library constructed in the vector lambda ZAP II (Stratagene) as per the manufacturer's instructions. Approximately 1.86×10^6 phages were initially plated and screened using a random-primer labeled portion of swine clone 7-3 (described above) as hybridization probe. Twenty one

20   positive clones were plaque purified. The inserts from these clones were excised from the bacteriophage into the phagemid pBluescript II SK- by co-infection with helper phage as described by the manufacturer (Stratagene). Human clones were characterized as has been described above for the swine clone.

25

EXAMPLE 11

DNA Encoding a Rat Growth Hormone Secretagogue Receptor (GHSR) Type Ia

30   Cross-hybridization under reduced stringency was the strategy utilized to isolate the rat GHSR type Ia. Approximately 10^6 phage plaques of a once-amplified rat pituitary cDNA library in lambda gt11 (RL1051b; Clontech, Palo Alto, CA) were plated on *E. coli* strain Y1090r-. The plaques were transferred to maximum-strength Nytran

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(Schleicher & Schuell, Keene, NH) denatured, neutralized and screened
with a 1.6 kb EcoRI/NotI fragment containing the entire coding and
untranslated regions of the swine GHSR, clone 7-3. The membranes
were incubated at 30°C in prehybridization solution (50% formamide, 2
5   X Denhardts, 5 X SSPE, 0.1% SDS, 100 μg/ml salmon sperm DNA) for
3 hours followed by overnight incubation in hybridization solution
(50% formamide, 2 X Denhardts, 5 X SSPE, 0.1% SDS, 10% dextran
sulfate, 100 μg/ml salmon sperm DNA) with 1 x 10^6 cpm/ml of [^{32}P]-
labeled probe. The probe was labeled with [^{32}P]dCTP using a random
10   priming kit (Gibco BRL, Gaithersburg, ND). After hybridization the
blots were washed two times each with 2 X SSC, 0.1% SDS (at 24°C,
then 37°C, and finally 55°C). A single positive clone was isolated
following three rounds of plaque purification. Phage containing the
GHSR was eluted from plate plaques with 1x lambda buffer (0.1M
15   NaCl, 0.01M MgSO4•7H2O, 35mM Tris-HCl, pH 7.5) following
overnight growth of approximately 200 pfu/150mm dish. After a ten
minute centrifugation at 10,000 x/g to remove debris, the phage solution
was treated with 1μg/ml RNAse A and DNAse I for thirty minutes at
24°C, followed by precipitation with 20% PEG (8000)/2M NaCl for two
20   hours on ice, and collection by centrifugation at 10,000 x/g for twenty
minutes. Phage DNA was isolated by incubation in 0.1% SDS, 30mM
EDTA, 50 μg/ml proteinase K for one hour at 68°C, with subsequent
phenol (three times) and chloroform (twice) extraction before
isopropanol precipitation overnight. The GHSR DNA insert (~6.4 kb)
25   was sub-cloned from lambda gt11 into the plasmid vector Litmus 28
(New England Biolabs, Beverly, MA). 2 μg of phage DNA was heated
to 65°C for ten minutes, then digested with 100 units BsiWI (New
England Biolab, Bevely, MA) at 37°C overnight. A 6.5 kb fragment
was gel purified, electroeluted and phenol/chloroform extracted prior to
30   ligation to BsiWI-digested Litmus 28 vector.

Double-stranded DNA was sequenced on both strands on a
ABI 373 automated sequencer using the ABI PRISM dye termination
cycle sequencing ready reaction kit (Perkin Elmer; Foster City, CA).

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For sequence comparisons and functional expression studies, a contiguous DNA fragment encoding the complete ORF (devoid of intervening sequence) for the rat GHSR type Ia was generated. The PCR was utilized to synthesize a amino-terminal
5 fragment from Met-1 to Val-260 with EcoRI (5') and HpaI (3') restriction sites appended, while a carboxyl-terminal fragment was generated from Lys-261 to Thr-364 with Dra I (5') and Not I (3') restriction sites appended. The ORF construct was assembled into the mammalian expression vector pSV7 via a three-way ligation with
10 EcoRI/Not I-digested pSV7, EcoRI/Hpa I-digested NH2-terminal fragment. and Dra I/Not I-digested C-terminal fragment.

Functional activity of the ORF construct was assessed by transfecting (using lipofectamine; GIBCO/BRL) 5 µg of plasmid DNA
15 into the aequorin expressing reporter cell line (293-AEQ17) cultured in 60 mm dishes. Following approximately 40 hours of expression the aequorin in the cells was charged for 2 hours with coelenterazine, the cells were harvested, washed and pelleted by low speed centrifugation into luminometer tubes. Functional activity was determined by
20 measuring Compound A dependent mobilization of intracellular calcium and concommitant calcium induced aequorin bioluminescence. Shown in Fig. 26 are three replicate samples exhibiting Compound A induced luminescent responses.

25 <u>EXAMPLE 12</u>

<u>Assays</u>
Mammalian cells (COS-7) were transfected with GHSR expression plasmids using Lipofectamine (GIBCO-BRL: Hawley-Nelson,
30 1993, *Focus* 15:73). Transfections were performed in 60 mm dishes on 80% confluent cells (approximately 4×10^5 cells) with 8 µg of Lipofectamine and 32 µg of GHSR plasmid DNA.
Binding of [^{35}S]-Compound A to swine pituitary membranes and crude membranes prepared from COS-7 cells

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transfected with GHSR expression plasmids was conducted. Crude cell membranes from COS-7 transfectants were prepared on ice, 48 hrs. post-transfection. Each 60 mm dish was washed twice with 3 ml of PBS, once with 1 ml homogenization buffer (50 mM Tris-HCl [pH 7.4],

5   5 mM $MgCl_2$, 2.5 mM EDTA, 30 μg/ml bacitracin). 0.5 ml of homogenization buffer was added to each dish, cells were removed by scraping and then homogenized using a Polytron device (Brinkmann, Syosset, NY; 3 bursts of 10 sec. at setting 4). The homogenate was then centrifuged for 20 min. at 11,000 x g at 0°C and the resulting crude

10   membrane pellet (chiefly containing cell membranes and nuclei) was resuspended in homogenization buffer supplemented with 0.06% BSA (0.1 ml/60 mm dish) and kept on ice. Binding reactions were performed at 20°C for 1 hr. in a total volume of 0.5 ml containing: 0.1 ml of membrane suspension, 10 μl of [^{35}S]-Compound A (0.05 to 1 nM;

15   specific activity approximately 900 Ci/mmol), 10 μl of competing drug and 380-390 μl of homogenization buffer. Bound radioligand was separated by rapid vacuum filtration (Brandel 48-well cell harvester) through GF/C filters pretreated for 1 hr. with 0.5% polyethylenimine. After application of the membrane suspension to the filter, the filters

20   were washed 3 times with 3 ml each of ice cold 50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 2.5 mM EDTA and 0.015% Triton X-100, and the bound radioactivity on the filers was quantitated by scintillation counting. Specific binding (> 90% of total) is defined as the difference between total binding and non-specific binding conducted in the

25   presence of 50 nM unlabeled Compound A.

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WHAT IS CLAIMED IS

1.   A method of determining the presence of an nucleic acid which encodes a growth hormone secretagogue receptor (GHSR) or a growth hormone related receptor (GHSRR) comprising:

a)   introducing a nucleic acid suspected of encoding a GHSR or GHSRR into a cell which does not naturally express the receptor on its cell membrane;

b)   introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a receptor-ligand binding event;

c)   contacting the cell with a growth hormone secretagogue; and

d)   determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

2.   A method to determine the presence of a growth hormone secretagogue comprising:

a)   introducing a nucleic acid which encodes a growth hormone secretagogue receptor into a cell under conditions so that growth hormone secretagogue receptor is expressed;

b)   introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand binding event;

c)   contacting the cell with a compound suspected of being a growth hormone secretagogue; and

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d) determining whether the compound is a growth hormone secretagogue by monitoring the detector molecule.

5   3.   A method to determine the presence of a nucleic acid which encodes a G protein cell membrane receptor comprising:

               a)   introducing at least one nucleic acid suspected of encoding a G protein cell membrane receptor into a cell;

10   b)   introducing a G-protein subunit into the cell;

               c)   introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule responds directly or indirectly to a G-protein receptor-

15   ligand binding event;

               d)   contacting the cell with a receptor ligand; and

               e)   determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

20   4.   A method according to Claim 3 wherein the cell does not naturally express the receptor on its cell membrane.

         5.   A method according to Claim 4 wherein the receptor is a growth hormone secretagogue receptor (GHSR).

25

         6.   A method according to Claim 4 wherein the receptor is a growth hormone secretagogue related receptor (GHSRR).

         7.   An assay to determine the presence of an nucleic acid

30 which encodes a growth hormone secretagogue receptor (GHSR) or a growth hormone secretagogue related receptor (GHSRR) comprising:

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a)   introducing at least one nucleic acid suspected
      of encoding a GHSR or GHSRR into a cell
      which does not naturally express the receptor
      on its cell membrane;

b)   introducing a G-protein subunit into the cell;

c)   introducing a detector molecule or a nucleic
      acid encoding a detector molecule into the cell,
      wherein the detector molecule is directly or
      indirectly responsive to a receptor-ligand
      binding event;

d)   contacting the cell with a growth hormone
      secretagogue; and

e)   determining whether the nucleic acid encodes a
      receptor by monitoring the detector molecule.

8.   A method according to Claim 7 wherein a pool
comprising at least 500 different nucleic acid molecules are introduced
into the cell in step a).

9.   A method according to Claim 8 wherein the pool
comprises RNA molecules.

10.   A method according to Claim 8 wherein upon
determining that the pool comprises a nucleic acid encoding a growth
hormone secretagogoue recptor is present, steps a) to e) are repeated,
except that the pool comprises a smaller number of different nucleic
acid molecules.

11.   A method according to Claim 10 wherein only one
type of oligonucleotide is introduced into the cell in step a).

12.   A method according to Claim 7 wherein the
G protein subunit is a G-alpha protein subunit.

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13.   A method according to Claim 12 wherein the G-protein subunit is the $G\alpha_{11}$ subunit.

14.   A method according to Claim 7 wherein the detector
5   molecule is aequorin.

15.   An assay method to determine the presence of a growth hormone secretagogue comprising:

  a)   introducing a nucleic acid which encodes a
10   growth hormone secretagogue receptor into a cell under conditions so that growth hormone secretagogue receptor is expressed;

  b)   introducing a G-protein subunit into the cell;

  c)   introducing a detector molecule or a nucleic
15   acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand binding event;

  d)   contacting the cell with a compound suspected
20   of being a growth hormone secretagogue; and

  e)   determining whether the compound is a growth hormone secretagogue by monitoring the detector molecule.

25   16.   A method according to Claim 15 wherein the G protein subunit is an G-alpha protein subunit.

17.   A method according to Claim 16 wherein the G-protein subunit is the $G\alpha_{11}$ subunit.

30

18.   A method according to Claim 15 wherein the result of step e) is compared to that obtained using a known growth hormone secretagogue.

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19.   An assay for identifying a ligand which binds to a human growth hormone secretagogue receptor comprising contacting a putative ligand with a human growth hormone secretagogue receptor in the presence of G protein subunit $\alpha11$ and determining whether binding

5   has occurred.

20.   An assay according to Claim 19 wherein the human growth hormone secretagogue receptor is expressed in a host cell which does not naturally express human growth hormone secretagogue

10   receptor.

21.   An assay according to Claim 20 wherein binding is detected by measuring the activity of a detector molecule.

15   22.   An assay according to Claim 21 wherein the detector molecule is aequorin.

23.   A ligand identified by the assay of any of Claims 15-22.

20   24.   A method of identifying a novel GHSR protein comprising exposing a labeled ligand to a putative GHSR protein and determining if binding occurs.

```
        10        20        30        40

CCTCACGCTGCCAGACCTGGGCTGGGACGCTCCCCCTGAA    40
AACGACTCGCTAGTGGAGGAGCTGCTGCCGCTCTTCCCCA    80
CGCCGCTGTTGGCGGGCGTCACCGCCACCTGCGTGGCGCT   120
CTTCGTGGTGGGTATCGCGGGCAACCTGCTCACGATGCTG   160
GTAGTGTCACGCTTCCGCGAGATGCGCACCACCACCAACC   200


       210       220       230       240

TCTACCTGTCCAGCATGGCCTTCTCCGACCTACTCATCTT   240
CCTCTGCATGCCCCTCGACCTCTTCCGCCTCTGGCAGTAC   280
CGGCCTTGGAACCTTGGCAACCTGCTCTGCAAACTCTTCC   320
AGTTCGTTAGCGAGAGCTGCACCTACGCCACAGTGCTCAC   360
CATCACCGCGCTGAGCGTCGAGCGCTACTTCGCCATCTGC   400


       410       420       430       440

TTCCCGCTGCGGGCCAAGGTAGTGGTCACCAAGGGCCGGG   440
TAAAGCTGGTCATCCTGGTCATCTGGGCCGTGGCCTTCTG   480
CAGCGCCGGGCCCATCTTCGTGCTGGTCGGAGTGGAGCAT   520
GATAACGGCACTGACCCTCGGGACACCAACGAGTGCCGCG   560
CCACGGAGTTCGCCGTGCGCTCCGGGCTGCTTACCGTCAT   600


       610       620       630       640

GGTCTGGGTGTCCAGTGTCTTCTTCTTCCTGCCTGTCTTC   640
TGCCTCACTGTGCTCTATAGCCTCATCGGCAGGAAGCTCT   680
GGCGGAGGAAGCGCGGCGAGGCGGCGGTGGGCTCCTCGCT   720
CAGGGACCAGAACCACAAACAAACCGTGAAAATGCTGGCT   760
GTAGTGGTGTTTGCTTTCATACTCTGCTGGCTGCCTTTCC   800


       810       820       830       840

ATGTAGGGCGATATTTATTTTCCAAATCCTTGGAGCCTGG   840
CTCTGTGGAGATTGCTCAGATCAGCCAATACTGCAACCTC   880
GTGTCCTTTGTCCTCTTCTACCTCAGTGCGGCCATCAACC   920
CTATTCTGTACAACATCATGTCCAAGAAGTATCGGGTGGC   960
GGTGTTCAAACTGCTGGGATTTGAGCCCTTCTCACAGAGG  1000


      1010      1020      1030      1040

AAACTCTCCACTCTGAAGGATGAAAGTTCTCGGGCCTGGA  1040
CAGAATCTAGTATTAATACATGA 1063
```

FIG.1

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```
              10          20
       MLVVSRFREM  RTTTNLYLSS    20
       MAFSDLLIFL  CMPLDLFRLW    40
       QYRPWNLGNL  LCKLFQFVSE    60
       SCTYATVLTI  TALSVERYFA    80
       ICFPLRAKVV  VTKGRVKLVI   100

              110         120
       LVIWAVAFCS  AGPIFVLVGV   120
       EHDNGTDPRD  TNECRATEFA   140
       VRSGLLTVMV  WVSSVFFFLP   160
       VFCLTVLYSL  IGRKLWRRKR   180
       GEAAVGSSLR  DQNHKQTVKM   200

              210         220
       LAVVVFAFIL  CWLPFHVGRY   220
       LFSKSLEPGS  VEIAQISQYC   240
       NLVSFVLFYL  SAAINPILYN   260
       IMSKKYRVAV  FKLLGFEPFS   280
       QRKLSTLKDE  SSRAWTESSI   300

              310         320

       NT 302
```

FIG.2

1   30
LTLPDLGWDA PPENDSLVEE LLPLFPTRLL

HELIX 1   60
AGVTATCVAL FVVGIAGNLL TMLVVSRFRE

        HELIX 2   90
MRTTTNLYLS SMAFSDLLIF ICMPLDLFRL

                        HELIX 3   120
WQYRPWNLGN LLCKLFQFVS ESCTYATVLT

                                    150
ITALSVERYF AICFPLRAKV VVTKGRVKLV

    HELIX 4   180
ILVIWAVAFC SAGPIFVLVG VEHDNGTDPR

                                    210
DTNECRATEF AVRSGLLTVM VWVSSVFFFL

HELIX 5   240
PVFCLTVLYS LIGRKLWRRK RGEAAVGSSL

                HELIX 6   270
RDQNHKQTVK MLAVVVFAFI LCWLPFHVGR

                                    300
YLFSKSLEPG SVEIAQISQY CNLVSFVLFY

HELIX 7   330
LSAAINPILY NIMSKKYRVA VFKLLGFEPF

                353
SQRKLSTLKD ESSRAWTESS INT

FIG.3

```
          10          20          30          40
GCAGCCTCTCACTTCCCTCTTTCCTCTCCTAGCATCCTCC       40
CTGAGAGCCCGCGCTCGATACTCCTTTGCACTCTTTCGCG       80
CCTAAGAGAACCTTCTCTGGGACCAGCCGGCTCCACCCTC      120
TCGGTCCTATCCAAGAGCCAGTTAAGCAGAGCCCTAAGCA      160
TGTGGAACGCGACCCCGAGCGAGGAACCGGGGCCCAACCT      200


          210         220         230         240
CACGCTGCCAGACCTGGGCTGGGACGCTCCCCCTGAAAAC      240
GACTCGCTAGTGGAGGAGCTGCTGCCGCTCTTCCCCACGC      280
CGCTGTTGGCGGGCGTCACCGCCACCTGCGTGGCGCTCTT      320
CGTGGTGGGTATCGCGGGCAACCTGCTCACGATGCTGGTA      360
GTGTCACGCTTCCGCGAGATGCGCACCACCACCAACCTCT      400


          410         420         430         440
ACCTGTCCAGCATGGCCTTCTCCGACCTACTCATCTTCCT      440
CTGCATGCCCCTCGACCTCTTCCGCCTTTGGCAGTACCGG      480
CCTTGGAACCTTGGCAACCTGCTCTGCAAACTCTTCCAGT      520
TCGTTAGCGAGAGCTGCACCTACGCCACAGTGCTCACCAT      560
CACCGCGCTGAGCGTCGAGCGCTACTTCGCCATCTGCTTC      600


          610         620         630         640
CCGCTGCGGGCCAAGGTAGTGGTCACCAAGGGCCGGGTAA      640
AGCTGGTCATCCTGGTCATCTGGGCCGTGGCCTTCTGCAG      680
CGCCGGGCCCATCTTCGTGCTGGTCGGAGTGGAGCATGAT      720
AACGGCACTGACCCTCGGGACACCAACGAGTGCCGCGCCA      760
CGGAGTTCGCCGTGCGCTCCGGGCTGCTTACCGTCATGGT      800


          810         820         830         840
CTGGGTGTCCAGTGTCTTCTTCTTCCTGCCTGTCTTCTGC      840
CTCACTGTGCTCTATAGCCTCATCGGCAGGAAGCTCTGGC      880
GGAGGAAGCGCGGCGAGGCGGCGGTGGGCTCCTCGCTCAG      920
GGACCAGAACCACAAACAAACCGTGAAAATGCTGGGTGGG      960
TCTCAATGCGCCCTCGAGCTTTCTCTCCCGGGTCCCCTCC     1000


         1010        1020        1030        1040
ACTCCTCGTGCCTTTTCTCTTCTCCCTGA 1029
```

FIG.4

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```
            10          20          30          40

MWNATPSEEP GPNLTLPDLG WDAPPENDSL VEELLPLFPT  40
PLLAGVTATC VALFVVGIAG NLLTMLVVSR FREMRTTTNL  80
YLSSMAFSDL LIFLCMPLDL FRLWQYRPWN LGNLLCKLFQ 120
FVSESCTYAT VLTITALSVE RYFAICFPLR AKVVVTKGRV 160
KLVILVIWAV AFCSAGPIFV LVGVEHDNGT DPRDTNECRA 200


            210         220         230         240

TEFAVRSGLL TVMVWVSSVF FFLPVFCLTV LYSLIGRKLW 240
RRKRGEAAVG SSLRDQNHKQ TVKMLGGSQC ALELSLPGPL 280
HSSCLFSSP  289
```

FIG.5

```
          10        20        30        40

CGCCCAGCGAAGAGCCGGGGTTCAACCTCACACTGGCCGA    40
CCTGGACTGGGATGCTTCCCCCGGCAACGACTCGCTGGGC    80
GACGAGCTGCTGCAGCTCTTCCCCGCGCCGCTGCTGGCGG   120
GCGTCACAGCCACCTGCGTGGCACTCTTCGTGGTGGGTAT   160
CGCTGGCAACCTGCTCACCATGCTGGTGGTGTCGCGCTTC   200


         210       220       230       240

CGCGAGCTGCGCACCACCACCAACCTCTACCTGTCCAGCA   240
TGGCCTTCTCCGATCTGCTCATCTTCCTCTGCATGCCCCT   280
GGACCTCGTTCGCCTCTGGCAGTACCGGCCCTGGAACTTC   320
GGCGACCTCCTCTGCAAACTCTTCCAATTCGTCAGTGAGA   360
GCTGCACCTACGCCACGGTGCTCACCATCACAGCGCTGAG   400


         410       420       430       440

CGTCGAGCGCTACTTCGCCATCTGCTTCCCACTCCGGGCC   440
AAGGTGGTGGTCACCAAGGGGCGGGTGAAGCTGGTCATCT   480
TCGTCATCTGGGCCGTGGCCTTCTGCAGCGCCGGGCCCAT   520
CTTCGTGCTAGTCGGGGTGGAGCACGAGAACGGCACCGAC   560
CCTTGGGACACCAACGAGTGCCGCCCCACCGAGTTTGCGG   600


         610       620       630       640

TGCGCTCTGGACTGCTCACGGTCATGGTGTGGGTGTCCAG   640
CATCTTCTTCTTCCTTCCTGTCTTCTGTCTCACGGTCCTC   680
TACAGTCTCATCGGCAGGAAGCTGTGGCGGAGGAGGCGCG   720
GCGATGCTGTCGTGGGTGCCTCGCTCAGGGACCAGAACCA   760
CAAGCAAACCGTGAAAATGCTGGCTGTAGTGGTGTTTGCC   800


         810       820       830       840

TTCATCCTCTGCTGGCTCCCCTTCCACGTAGGGCGATATT   840
TATTTTCCAAATCCTTTGAGCCTGGCTCCTTGGAGATTGC   880
TCAGATCAGCCAGTACTGCAACCTCGTGTCCTTTGTCCTC   920
TTCTACCTCAGTGCTGCCATCAACCCCATTCTGTACAACA   960
TCATGTCCAAGAAGTACCGGGTGGCAGTGTTCAGACTTCT  1000


        1010      1020      1030      1040

GGGATTCGAACCCTTCTCCCAGAGAAAGCTCTCCACTCTG  1040
AAAGATGAAAGTTCTCGGGCCTGGACAGAATCTAGTATTA  1080
ATACATGA  1088
```

FIG.6

```
              10              20

       MLVVSRFREL   RTTTNLYLSS    20
       MAFSDLLIFL   CMPLDLVRLW    40
       QYRPWNFGDL   LCKLFQFVSE    60
       SCTYATVLTI   TALSVERYFA    80
       ICFPLRAKVV   VTKGRVKLVI   100


              110             120

       FVIWAVAFCS   AGPIFVLVGV   120
       EHENGTDPWD   TNECRPTEFA   140
       VRSGLLIVMV   WVSSIIIILP   160
       VFCLTVLYSL   IGRKLWRRRR   180
       GDAVVGASLR   DQNHKQTVKM   200


              210             220

       LAVVVFAFIL   CWLPFHVGRY   220
       LFSKSFEPGS   LEIAQISQYC   240
       NLVSFVLFYL   SAAINPILYN   260
       IMSKKYRVAV   FRLLGFEPFS   280
       QRKLSTLKDE   SSRAWTESSI   300


              310             320

       NT  302
```

FIG.7

●　●　

```
1                                          30
PSEEPGFNLT  LADLDWDASP  GNDSLGDELL


                              HELIX 1    60
QLFPAP LLAG  VTATCVALFV  VGIAGNLLTM


                              HELIX 2    90
LVVSRFRELR  TTTNLYLSSM  AFSDLLIFL C


                                         120
MPLDLVRLWQ  YRPWNFGDLL  CK LFQFVSES

HELIX 3                                  150
CTYATVLTIT  ALSV ERYD AI  CFPLRAKVVV


                         HELIX 4        180
TKGRVK LVIF  VIWAVAFCSA  GPIFVLVGV E


                                        210
HENGTDPWDT  NECRPTEFAV  R SGLLTVMVW


       HELIX 5                          240
VSSIFFFLPV  FCLTVLYSLI  G RKLWRRRRG


                              HELIX 6 270
DAVVGASLRD  QNHKQT VKML  AVVVFAFILC


                                        300
WLPFHVG RYL  FSKS FEPGSL  EIAQISQYCN


          HELIX 7                       330
LVSFVLFYLS  AAINPILYNI  MS KKYRVAVF


                                        360
RLLGFEPFSQ  RKLSTLKDES  SRAWTESSIN


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T
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FIG.8

```
        10          20          30          40
         .           .           .           .
GCGCCTCACGCTCCCGCTTCGCGGCGCCTGGTCCCTGCGG          40
TCCCCACTCGCTGCGACGCTTTGGGAAGTGCGAGATGGAA          80
CTGGATCGAGAACGCAAATGCGAGGCAGGGCTGGTGACAG          120
CATCCTCCCTACGCGTCTGCACCCGCTCCTCCCTCGCACC          160
CTCCCGCGCCTAAGCGGACCTCCTCGGGAGCCAGCTCGGT          200


        210         220         230         240
         .           .           .           .
CCAGCCTCCCAGCGCAGTCACGTCCCAGAGCCTGTTCAGC          240
TGAGCCGGCAGCATGTGGAACGCGACGCCCAGCGAAGAGC          280
CGGGGTTCAACCTCACACTGGCCGACCTGGACTGGGATGC          320
TTCCCCCGGCAACGACTCGCTGGGCGACGAGCTGCTGCAG          360
CTCTTCCCCGCGCCGCTGCTGGCGGGCGTCACAGCCACCT          400


        410         420         430         440
         .           .           .           .
GCGTGGCACTCTTCGTGGTGGGTATCGCTGGCAACCTGCT          440
CACCATGCTGGTGGTGTCGCGCTTCCGCGAGCTGCGCACC          480
ACCACCAACCTCTACCTGTCCAGCATGGCCTTCTCCGATC          520
TGCTCATCTTCCTCTGCATGCCCCTGGACCTCGTTCGCCT          560
CTGGCAGTACCGGCCCTGGAACTTCGGCGACCTCCTCTGC          600


        610         620         630         640
         .           .           .           .
AAACTCTTCCAATTCGTCAGTGAGAGCTGCACCTACGCCA          640
CGGTGCTCACCATCACAGCGCTGAGCGTCGAGCGCTACTT          680
CGCCATCTGCTTCCCACTCCGGGGCCAAGGTGGTGGTCACC          720
AAGGGGCGGGTGAAGCTGGTCATCTTCGTCATCTGGGCCG          760
TGGCCTTCTGCAGCGCCGGGCCCATCTTCGTGCTAGTCGG          800


        810         820         830         840
         .           .           .           .
GGTGGAGCACGAGAACGGCACCGACCCTTGGGACACCAAC          840
GAGTGCCGCCCCACCGAGTTTGCGGTGCGCTCTGGACTGC          880
TCACGGTCATGGTGTGGGTGTCCAGCATCTTCTTCTTCCT          920
TCCTGTCTTCTGTCTCACGGTCCTCTACAGTCTCATCGGC          960
AGGAAGCTGTGGCGGAGGAGGCGCGGCGATGCTGTCGTGG          1000
```

FIG.9A

●

●

```
        1010        1020        1030        1040

GTGCCTCGCTCAGGGACCAGAACCACAAGCAAACCGTGAA  1040
AATGCTGGGTGGGTCTCAGCGCGCGCTCAGGCTTTCTCTC  1080
GCGGGTCCTATCCTCTCCCTGTGCCTTCTCCCTTCTCTCT  1120
GA  1122
```

FIG.9B

```
       10          20          30          40

MWNATPSEEPGFNLTLADLDWDASPGNDSLGDELLQLFPA    40
PLLAGVTATCVALFVVGIAGNLLTMLVVSRFRELRTTTNL    80
YLSSMAFSDLLIFLCMPLDLVRLWQYRPWNFGDLLCKLFQ   120
FVSESCTYATVLTITALSVERYFAICFPLRAKVVVTKGRV   160
KLVIFVIWAVAFCSAGPIFVLVGVEHENGTDPWDTNECRP   200


       210         220         230         240

TEFAVRSGLLTVMVWVSSIFFFLPVFCLTVLYSLIGRKLW   240
RRRRGDAVVGASLRDQNHKQTVKMLGGSQRALRLSLAGPI   280
LSLCLLPSL   289
```

FIG.10

```
       10          20          30          40

MPLDLVRLWQYRPWNFGDLLCKLFQFVSESCTYATVLTIT    40
ALSVERYFAICFPLRAKVVVTKGRVKLVIFVIWAVAFCSA    80
GPIFVLVGVEHENGTDPWDTNECRPTEFAVRSGLLTVMVW   120
VSSIFFFLPVFCLTVLYSLIGRKLWRRRRGDAVVGASLRD   160
QNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKSFEPGSL   200


       210         220         230         240

EIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVAVF   240
RLLGFEPFSQRKLSTLKDESSRAWTESSINT   271
```

FIG.12

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<pre>
      10   20   30   40

ATCTGCTCATCTTCCTCTGCATGCCCCTGGACCTCGTTCG   40
CCTCTGGCAGTACCGGCCCTGGAACTTCGGCGACCTCCTC   80
TGCAAACTCTTCCAATTCGTCAGTGAGAGCTGCACCTACG   120
CCACGGTGCTCACCATCACAGCGCTGAGCGTCGAGCGCTA   160
CTTCGCCATCTGCTTCCCACTCCGGGCCAAGGTGGTGGTC   200

     210   220   230   240

ACCAAGGGGCGGGTGAAGCTGGTCATCTTCGTCATCTGGG   240
CCGTGGCCTTCTGCAGCGCCGGGCCCATCTTCGTGCTAGT   280
CGGGGTGGAGCACGAGAACGGCACCGACCCTTGGGACACC   320
AACGAGTGCCGCCCCACCGAGTTTGCGGTGCGCTCTGGAC   360
TGCTCACGGTCATGGTGTGGGTGTCCAGCATCTTCTTCTT   400

     410   420   430   440

CCTTCCTGTCTTCTGTCTCACGGTCCTCTACAGTCTCATC   440
GGCAGGAAGCTGTGGCGGAGGAGGCGCGGCGATGCTGTCG   480
TGGGTGCCTCGCTCAGGGACCAGAACCACAAGCAAACCGT   520
GAAAATGCTGGCTGTAGTGGTGTTTGCCTTCATCCTCTGC   560
TGGCTCCCCTTCCACGTAGGGCGATATTTATTTTCCAAAT   600

     610   620   630   640

CCTTTGAGCCTGGCTCCTTGGAGATTGCTCAGATCAGCCA   640
GTACTGCAACCTCGTGTCCTTTGTCCTCTTCTACCTCAGT   680
GCTGCCATCAACCCCATTCTGTACAACATCATGTCCAAGA   720
AGTACCGGGTGGCAGTGTTCAGACTTCTGGGATTCGAACC   760
CTTCTCCCAGAGAAAGCTCTCCACTCTGAAAGATGAAAGT   800

   810   820   830   840

TCTCGGGCCTGGACAGAATCTAGTATTAATACATGA   836
</pre>

FIG.11

```
                                                    v10         v20
FIG.3-SWINE TYPE I CLONE 7-3orf      LTLPDLGWDAPPENDSLVEE
                                     LTLPDLGWDAPPENDSLVEE
FIG.5-SWINE TYPE II CLONE 1375m      LTLPDLGWDAPPENDSLVEE
                                          ^20         ^30

                                                    v30         v40
FIG.3-SWINE TYPE I CLONE 7-3orf      LLPLFPTPLLAGVTATCVAL
                                     LLPLFPTPLLAGVTATCVAL
FIG.5-SWINE TYPE II CLONE 1375m      LLPLFPTPLLAGVTATCVAL
                                          ^40         ^50

                                                    v50         v60
FIG.3-SWINE TYPE I CLONE 7-3orf      FVVGIAGNLLTMLVVSRFRE
                                     FVVGIAGNLLTMLVVSRFRE
FIG.5-SWINE TYPE II CLONE 1375m      FVVGIAGNLLTMLVVSRFRE
                                          ^60         ^70

                                                    v70         v80
FIG.3-SWINE TYPE I CLONE 7-3orf      MRTTTNLYLSSMAFSDLLIF
                                     MRTTTNLYLSSMAFSDLLIF
FIG.5-SWINE TYPE II CLONE 1375m      MRTTTNLYLSSMAFSDLLIF
                                          ^80         ^90

                                                    v90        v100
FIG.3-SWINE TYPE I CLONE 7-3orf      LCMPLDLFRLWQYRPWNLGN
                                     LCMPLDLFRLWQYRPWNLGN
FIG.5-SWINE TYPE II CLONE 1375m      LCMPLDLFRLWQYRPWNLGN
                                          ^100        ^110

                                                   v110        v120
FIG.3-SWINE TYPE I CLONE 7-3orf      LLCKLFQFVSESCTYATVLT
                                     LLCKLFQFVSESCTYATVLT
FIG.5-SWINE TYPE II CLONE 1375m      LLCKLFQFVSESCTYATVLT
                                          ^120        ^130

                                                   v130        v140
FIG.3-SWINE TYPE I CLONE 7-3orf      ITALSVERYFAICFPLRAKV
                                     ITALSVERYFAICFPLRAKV
FIG.5-SWINE TYPE II CLONE 1375m      ITALSVERYFAICFPLRAKV
                                          ^140        ^150

                                                   v150        v160
FIG.3-SWINE TYPE I CLONE 7-3orf      VVTKGRVKLVILVIWAVAFC
                                     VVTKGRVKLVILVIWAVAFC
FIG.5-SWINE TYPE II CLONE 1375m      VVTKGRVKLVILVIWAVAFC
                                          ^160        ^170
```

FIG.13A

```
                                                      v170        v180
FIG.3-SWINE TYPE I CLONE 7-3orf   SAGPIFVLVGVEHDNGTDPR
                                  SAGPIFVLVGVEHDNGTDPR
FIG.5-SWINE TYPE II CLONE 1375m   SAGPIFVLVGVEHDNGTDPR
                                       ^180        ^190

                                                      v190        v200
FIG.3-SWINE TYPE I CLONE 7-3orf   DTNECRATEFAVRSGLLTVM
                                  DTNECRATEFAVRSGLLTVM
FIG.5-SWINE TYPE II CLONE 1375m   DTNECRATEFAVRSGLLTVM
                                       ^200        ^210

                                                      v210        v220
FIG.3-SWINE TYPE I CLONE 7-3orf   VWVSSVFFFLPVFCLTVLYS
                                  VWVSSVFFFLPVFCLTVLYS
FIG.5-SWINE TYPE II CLONE 1375m   VWVSSVFFFLPVFCLTVLYS
                                       ^220        ^230

                                                      v230        v240
FIG.3-SWINE TYPE I CLONE 7-3orf   LIGRKLWRRKRGEAAVGSSL
                                  LIGRKLWRRKRGEAAVGSSL
FIG.5-SWINE TYPE II CLONE 1375m   LIGRKLWRRKRGEAAVGSSL
                                       ^240        ^250

                                                      v250        v260
FIG.3-SWINE TYPE I CLONE 7-3orf   RDQNHKQTVKMLAVVVFAFI
                                  RDQNHKQTVKML:    A:
FIG.5-SWINE TYPE II CLONE 1375m   RDQNHKQTVKMLGGSQCALE
                                       ^260        ^270

                                                      v270
FIG.3-SWINE TYPE I CLONE 7-3orf   LCWL-PFHVGRYLFSKS
                                  L.   P:H :..LFS.:
FIG.5-SWINE TYPE II CLONE 1375m   LSLPGPLH-SSCLFSSP
                                       ^280
```

FIG.13B

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v10        v20
PSEEPGFNLTLADLDWDASP
PSEEPGFNLTLADLDWDASP
PSEEPGFNLTLADLDWDASP
      ^10        ^20
```

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v30        v40
GNDSLGDELLQLFPAPLLAG
GNDSLGDELLQLFPAPLLAG
GNDSLGDELLQLFPAPLLAG
      ^30        ^40
```

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v50        v60
VTATCVALFVVGIAGNLLTM
VTATCVALFVVGIAGNLLTM
VTATCVALFVVGIAGNLLTM
      ^50        ^60
```

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v70        v80
LVVSRFRELRTTTNLYLSSM
LVVSRFRELRTTTNLYLSSM
LVVSRFRELRTTTNLYLSSM
      ^70        ^80
```

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v90        v100
AFSDLLIFLCMPLDLVRLWQ
AFSDLLIFLCMPLDLVRLWQ
AFSDLLIFLCMPLDLVRLWQ
      ^90        ^100
```

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v110       v120
YRPWNFGDLLCKLFQFVSES
YRPWNFGDLLCKLFQFVSES
YRPWNFGDLLCKLFQFVSES
      ^100       ^110
```

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v130       v140
CTYATVLTITALSVERYFAI
CTYATVLTITALSVERYFAI
CTYATVLTITALSVERYFAI
      ^130       ^140
```

FIG.8-HUMAN TYPE I 1146orf

FIG.10-HUMAN TYPE II CLONE1141m

```
              v150       v160
CFPLRAKVVVTKGRVKLVIF
CFPLRAKVVVTKGRVKLVIF
CFPLRAKVVVTKGRVKLVIF
      ^150       ^160
```

FIG.14A

	v170 v180
FIG.8-HUMAN TYPE I 1146orf	VIWAVAFCSAGPIFVLVGVE
	VIWAVAFCSAGPIFVLVGVE
FIG.10-HUMAN TYPE II CLONE1141m	VIWAVAFCSAGPIFVLVGVE
	^170 ^180

	v190 v200
FIG.8-HUMAN TYPE I 1146orf	HENGTDPWDTNECRPTEFAV
	HENGTDPWDTNECRPTEFAV
FIG.10-HUMAN TYPE II CLONE1141m	HENGTDPWDTNECRPTEFAV
	^190 ^200

	v210 v220
FIG.8-HUMAN TYPE I 1146orf	RSGLLTVMVWVSSIFFFLPV
	RSGLLTVMVWVSSIFFFLPV
FIG.10-HUMAN TYPE II CLONE1141m	RSGLLTVMVWVSSIFFFLPV
	^210 ^220

	v230 v240
FIG.8-HUMAN TYPE I 1146orf	FCLTVLYSLIGRKLWRRRG
	FCLTVLYSLIGRKLWRRRG
FIG.10-HUMAN TYPE II CLONE1141m	FCLTVLYSLIGRKLWRRRG
	^230 ^240

	v250 v260
FIG.8-HUMAN TYPE I 1146orf	DAVVGASLRDQNHKQTVKML
	DAVVGASLRDQNHKQTVKML
FIG.10-HUMAN TYPE II CLONE1141m	DAVVGASLRDQNHKQTVKML
	^250 ^260

FIG.14B

```
                                          v10       v20        v30        v40
FIG.3-SWINE TYPE I CLONE 7-3orf   LTLPDLGWDAPPENDSLVEELLPLFPTPLLAGVTATCVAL
                                  LTL:DL:WDA:P.NDSL  :ELL.LFP:PLLAGVTATCVAL
FIG.8-HUMAN TYPE I 1146orf        LTLADLDWDASPGNDSLGDELLQLFPAPLLAGVTATCVAL
                                   ^10      ^20        ^30        ^40
                                          v50       v60        v70        v80
FIG.3-SWINE TYPE I CLONE 7-3orf   FVVGIAGNLLTMLVVSRFREMRTTTNLYLSSMAFSDLLIF
                                  FVVGIAGNLLTMLVVSRFRE:RTTTNLYLSSMAFSDLLIF
FIG.8-HUMAN TYPE I 1146orf        FVVGIAGNLLTMLVVSRFRELRTTTNLYLSSMAFSDLLIF
                                   ^50      ^60        ^70        ^80
                                          v90       v100       v110       v120
FIG.3-SWINE TYPE I CLONE 7-3orf   LCMPLDLFRLWQYRPWNLGNLLCKLFQFVSESCTYATVLT
                                  LCMPLDL RLWQYRPWN:G:LLCKLFQFVSESCTYATVLT
FIG.8-HUMAN TYPE I 1146orf        LCMPLDLVRLWQYRPWNFGDLLCKLFQFVSESCTYATVLT
                                   ^90      ^100       ^110       ^120
                                         v130      v140       v150       v160
FIG.3-SWINE TYPE I CLONE 7-3orf   ITALSVERYFAICFPLRAKVVVTKGRVKLVILVIWAVAFC
                                  ITALSVERYFAICFPLRAKVVVTKGRVKLVI:VIWAVAFC
FIG.8-HUMAN TYPE I 1146orf        ITALSVERYFAICFPLRAKVVVTKGRVKLVIFVIWAVAFC
                                   ^130     ^140       ^150       ^160
                                         v170      v180       v190       v200
FIG.3-SWINE TYPE I CLONE 7-3orf   SAGPIFVLVGVEHDNGTDPRDTNECRATEFAVRSGLLTVM
                                  SAGPIFVLVGVEH:NGTDP:DTNECR:TEFAVRSGLLTVM
FIG.8-HUMAN TYPE I 1146orf        SAGPIFVLVGVEHENGTDPWDTNECRPTEFAVRSGLLTVM
                                   ^170     ^180       ^190       ^200
                                         v210      v220       v230       v240
FIG.3-SWINE TYPE I CLONE 7-3orf   VWVSSVFFFLPVFCLTVLYSLIGRKLWRRKRGEAAVGSSL
                                  VWVSS:FFFLPVFVLTVLYSLIGRKLWRR:RG:A.VG:SL
FIG.8-HUMAN TYPE I 1146orf        VWVSSIFFFLPVFCLTVLYSLIGRKLWRRRRGDAVVGASL
                                   ^210     ^220       ^230       ^240
                                         v250      v260       v270       v280
FIG.3-SWINE TYPE I CLONE 7-3orf   RDQNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKSLEPG
                                  RDQNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKS:EPG
FIG.8-HUMAN TYPE I 1146orf        RDQNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKSFEPG
                                   ^250     ^260       ^270       ^280
                                         v290      v300       v310       v320
FIG.3-SWINE TYPE I CLONE 7-3orf   SVEIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVA
                                  S:EIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVA
FIG.8-HUMAN TYPE I 1146orf        SLEIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVA
                                   ^290     ^300       ^310       ^320
                                         v330      v340       v350
FIG.3-SWINE TYPE I CLONE 7-3orf   VFKLLGFEPFSQRKLSTLKDESSRAWTESSINT
                                  VF:LLGFEPFSQRKLSTLKDESSRAWTESSINT
FIG.8-HUMAN TYPE I 1146orf        VFRLLGFEPFSQRKLSTLKDESSRAWTESSINT
                                   ^330     ^340       ^350       ^360
```

FIG.15

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

FIG.5-SWINE TYPE II CLONE 1375m

FIG.10-HUMAN TYPE II CLONE1141m

```
                        v10       v20
MWNATPSEEPGPNLTLPDLG
MWNATPSEEPG NLTL:DL:
MWNATPSEEPGFNLTLADLD
              ^10       ^20
                        v30       v40
WDAPPENDSLVEELLPLFPT
WDA:P.NDSL :ELL.LFP:
WDASPGNDSLGDELLQLFPA
              ^30       ^40
                        v50       v60
PLLAGVTATCVALFVVGIAG
PLLAGVTATCVALFVVGIAG
PLLAGVTATCVALFVVGIAG
              ^50       ^60
                        v70       v80
NLLTMLVVSRFREMRTTTNL
NLLTMLVVSRFRE:RTTTNL
NLLTMLVVSRFRELRTTTNL
              ^70       ^80
                        v90       v100
YLSSMAFSDLLIFLCMPLDL
YLSSMAFSDLLIFLCMPLDL
YLSSMAFSDLLIFLCMPLDL
              ^90       ^100
                        v110      v120
FRLWQYRPWNLGNLLCKLFQ
RLWQYRPWN:G:LLCKLFQ
VRLWQYRPWNFGDLLCKLFQ
              ^110      ^120
                        v130      v140
FVSESCTYATVLTITALSVE
FVSESCTYATVLTITALSVE
FVSESCTYATVLTITALSVE
              ^130      ^140
                        v150      v160
RYFAICFPLRAKVVVTKGRV
RYFAICFPLRAKVVVTKGRV
RYFAICFPLRAKVVVTKGRV
              ^150      ^160
```

FIG.16A

```
                                                          v170        v180
FIG.5-SWINE TYPE II CLONE 1375m        KLVILVIWAVAFCSAGPIFV
                                       KLVI:VIWAVAFCSAGPIFV
FIG.10-HUMAN TYPE II CLONE1141m        KLVIFVIWAVAFCSAGPIFV
                                                          ^170        ^180
                                                          v190        v200
FIG.5-SWINE TYPE II CLONE 1375m        LVGVEHDNGTDPRDTNECRA
                                       LVGVEH:NGTDP:DTNECR:
FIG.10-HUMAN TYPE II CLONE1141m        LVGVEHENGTDPWDTNECRP
                                                          ^190        ^200
                                                          v210        v220
FIG.5-SWINE TYPE II CLONE 1375m        TEFAVRSGLLTVMVWVSSVI
                                       TEFAVRSGLLTVMVWVSS:F
FIG.10-HUMAN TYPE II CLONE1141m        TEFAVRSGLLTVMVWVSSIF
                                                          ^210        ^220
                                                          v230        v240
FIG.5-SWINE TYPE II CLONE 1375m        FFLPVFCLTVLYSLIGRKLW
                                       FFLPVFCLTVLYSLIGRKLW
FIG.10-HUMAN TYPE II CLONE1141m        FFLPVFCLTVLYSLIGRKLW
                                                          ^230        ^240
                                                          v250        v260
FIG.5-SWINE TYPE II CLONE 1375m        RRKRGEAAVGSSLRDQNHKQ
                                       RR:RG:A.VG:SLRDQNHKQ
FIG.10-HUMAN TYPE II CLONE1141m        RRRRGDAVVGASLRDQNHKQ
                                                          ^250        ^260
                                                          v270        v280
FIG.5-SWINE TYPE II CLONE 1375m        TVKMLGGSQCALELSLPGPL
                                       TVKMLGGSQ AL LSL:GP:
FIG.10-HUMAN TYPE II CLONE1141m        TVKMLGGSQRALRLSLAGPI
                                                          ^270        ^280

FIG.5-SWINE TYPE II CLONE 1375m        HSSCLFSS
                                       S CL::S
FIG.10-HUMAN TYPE II CLONE1141m        LSLCLLPS
```
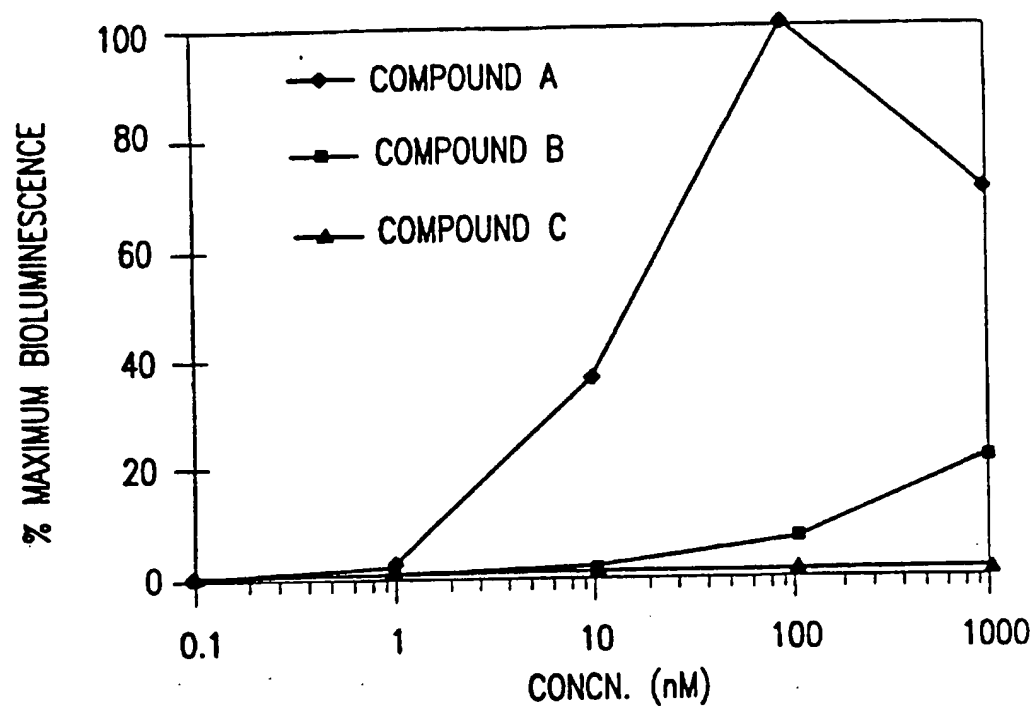
FIG.16B

FIG.17
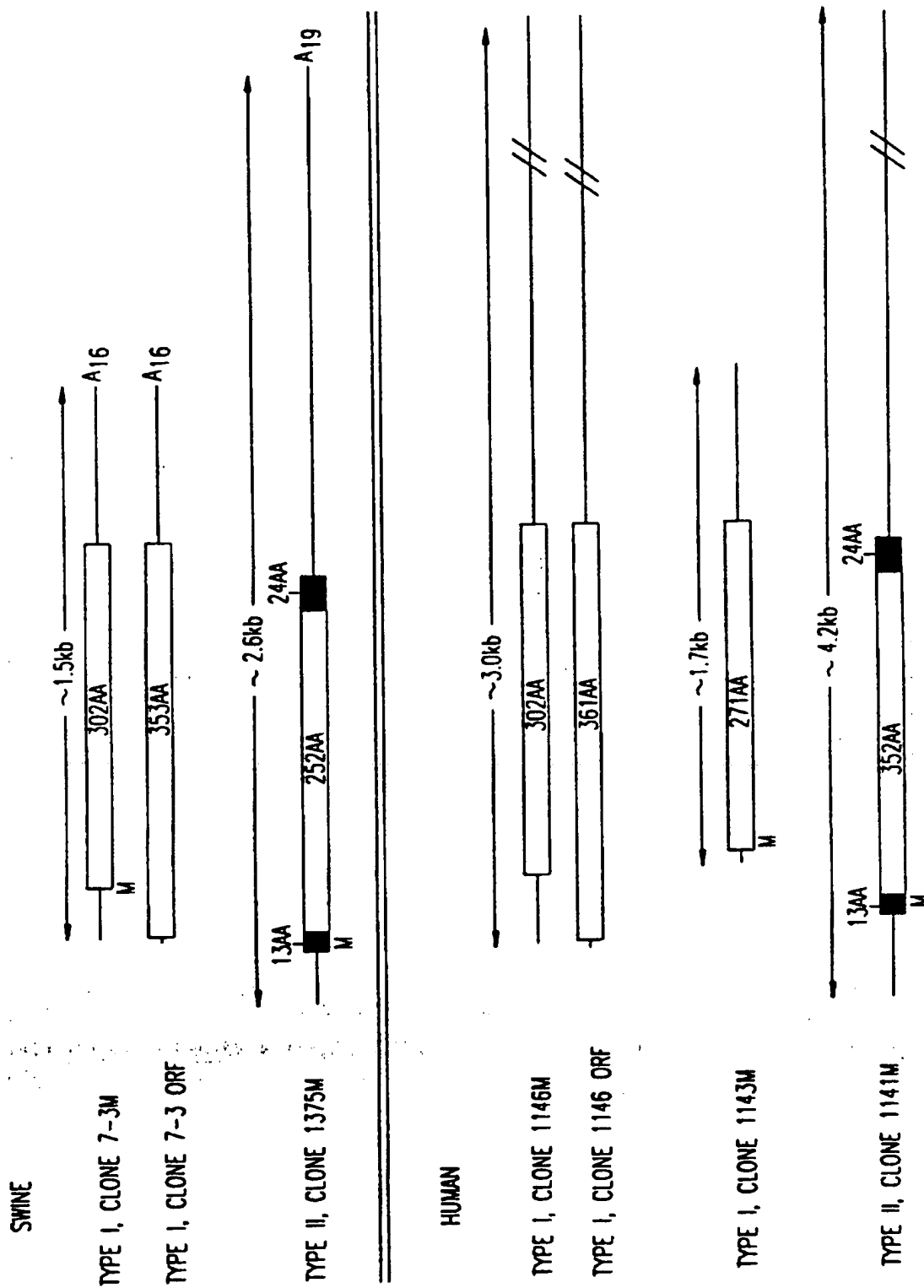
FIG.18

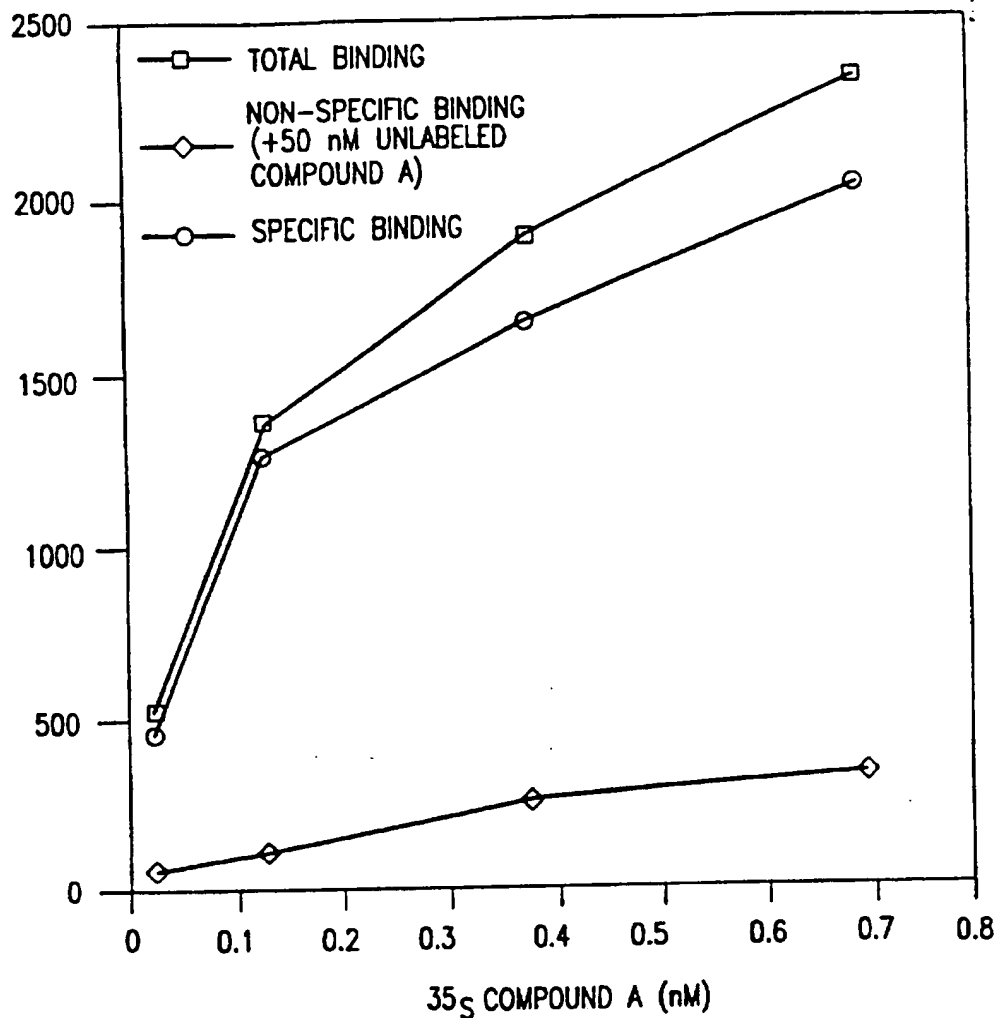| (100Nm)	SWINE CLONE 7-3		HUMAN CLONE 1146	
	24 HOURS	48 HOURS	24 HOURS	48 HOURS
COMPOUND A (100nm) (1000 nM)	13,553 9,176	2,692	1,353 3,091	2,228
COMPOUND B (100nM) COMPOUND C (100nM)	717 100	425 58	113 96	108 67
GHRP-2 (1000 nM) GHRP-6 (1000 nM)	2,492 5,003		1542 617	

FIG.19

FIG.20

Legend:
- □ TOTAL BINDING
- ◇ NON-SPECIFIC BINDING (+50 nM UNLABELED COMPOUND A)
- ○ SPECIFIC BINDING

x-axis: ^{35}S COMPOUND A (nM)

LIGAND	INHIBITION (% OF CONTROL SPECIFIC BINDING)
COMPOUND A @ 5nM	97
GHRP-6 @ 10nM	84
COMPOUND C	
1.692,428 @ 1 μM	43
GALAMIN @ 10 μM	44
AMENOMEDIN N @ 10 μM	19

FIG.21

```
  1   MWNATPSEEP GFNLTLADLD WDASPGNDSL GDELLQLFPA PLLAGVTATC

 51   VALFVVGIAG NLLTMLVVSR FRELRTTTNL YLSSMAFSDL LIFLCMPLDL

101   VRLWQYRPWN FGDLLCKLFQ FVSESCTYAT VLTITALSVE RYFAICFPLR

151   AKVVVTKGRV KLVIFVIWAV AFCSAGPIFV LVGVEHENGT DPWDTNECRP

201   TEFAVRSGLL TVMVWVSSIF FFLPVFCLTV LYSLIGRKLW RRRRGDAVVG

251   ASLRDQNHKQ TVKMLAVVVF AFILCWLPFH VGRYLFSKSF EPGSLEIAQI

301   SQYCNLVSFV LFYLSAAINP ILYNIMSKKY RVAVFRLLGF EPFSQRKLST

351   LKDESSRAWT ESSINT*
```

FIG.22
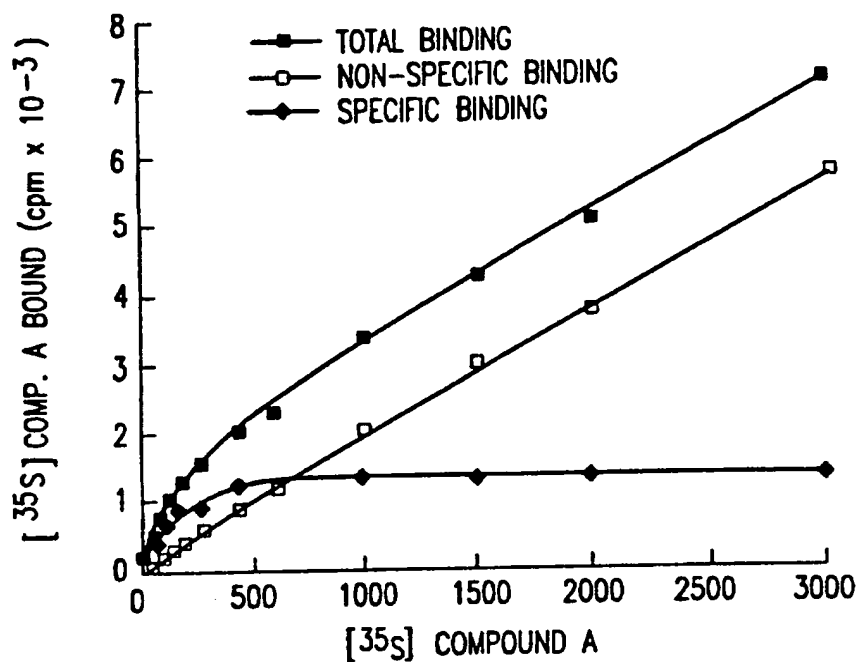
FIG.23A
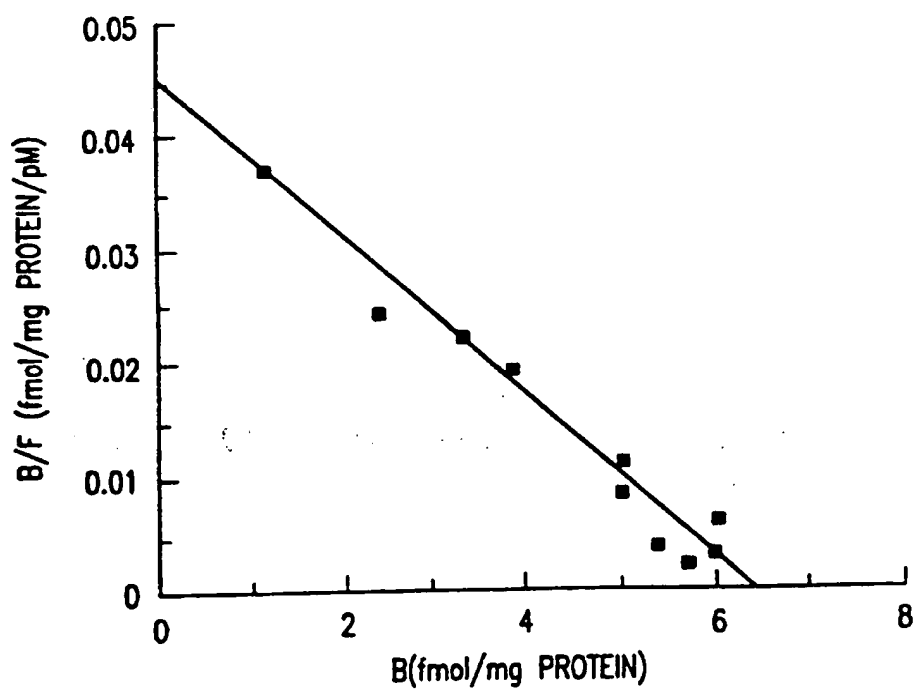
FIG.23B
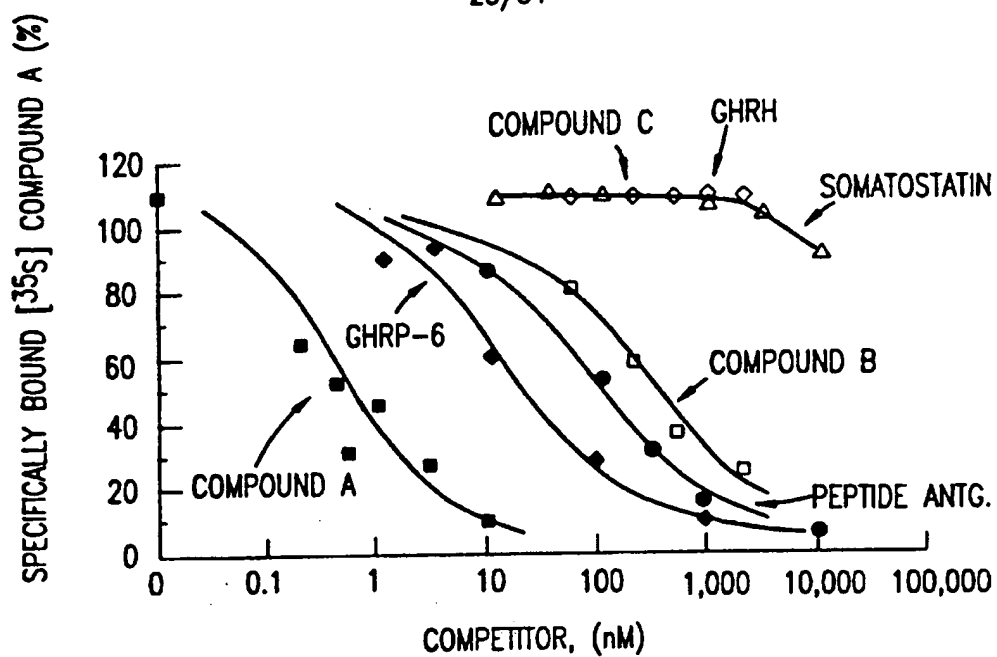
FIG.24
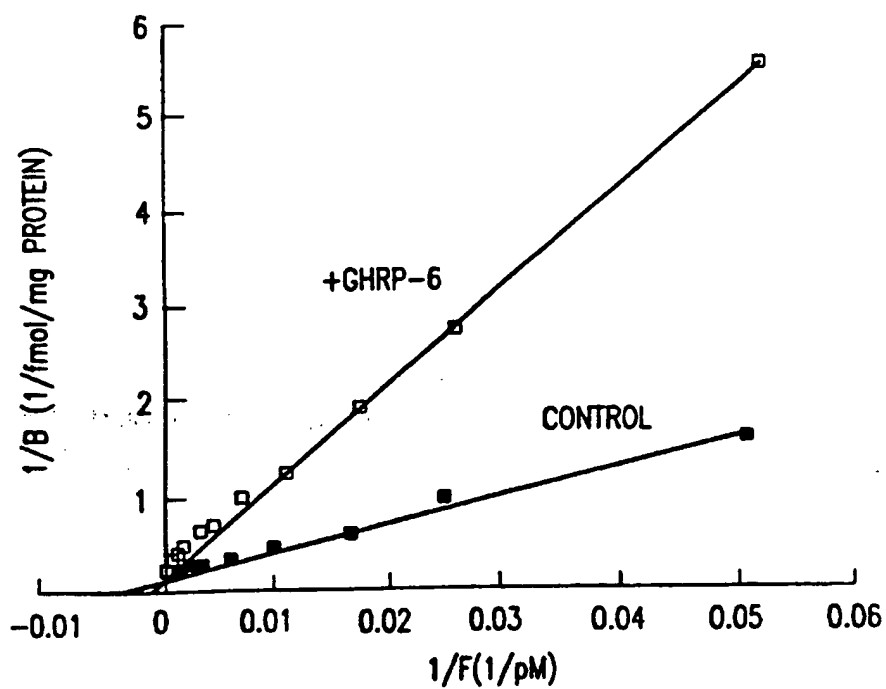
FIG.25
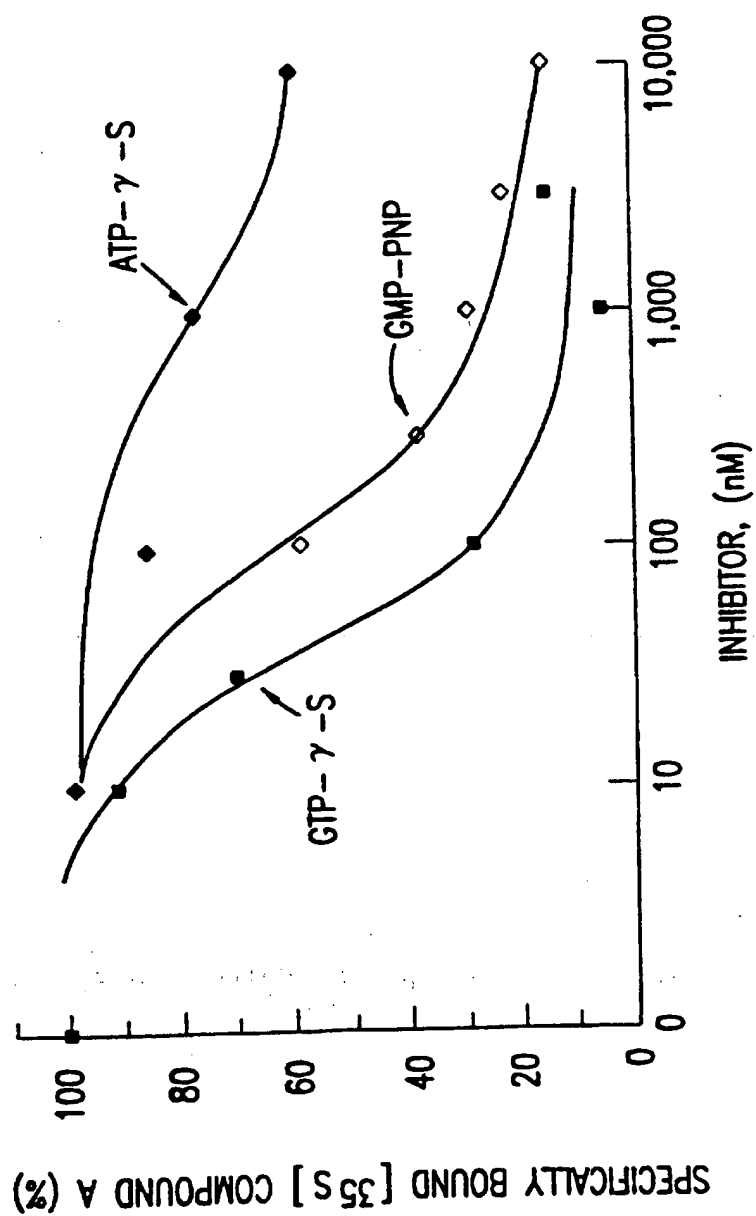
FIG.26

```
          10          20          30          40          50          60
ATG TGG AAC GCG ACC CCC AGC GAG GAG CCG GAG CCT AAC GTC ACG TTG GAC CTG GAT TGG    60
GAC GCT TCC CCC GGC AAC GAC TCA CTG CCT GAC GAA CTG CTG CCG TTC CCC GCT CCG AAC   120
CTG CTG GCA GGC GTC ACC GCC ACC GCC TGC TTC CGC GAG CTC CGC ATC TCA GGC CTC TAC   180
CTG CTC ACT ATG CTG GTG TCC TTC CGC GAG CTG CGC GAG ATC TTC GAC ACC AAC CTC GTC   240
CTG TCC AGC ATG GCC GAT CTG CTG ATG TGC CTG ATG CCG CTG GAC CTG GAC CTC GTC       300

         310         320         330         340         350         360
CGC CTC TGG CAG TAC CGG CCC TGG AAC TTC GGC GAC CTG CTC TGC AAA CTC TTC CAG TTT   360
GTC AGC GAG AGC TGC ACC TAC TGC GCC ATC ACC GTG GTG AGC GTC GCG CTG AGC GTG CGC   420
TAC TTC GCC ATC TGC CCT CTG CGG GCC CTG GTG GCT ACT GGG CCC ATC TTC GTG GTG CTG   480
CTG GTC ATC CTT GTC GCC ATC TGG GCC GTG GTG GCG CCC ATC TTC TGC CGC GCC ACC       540
GTG GGC GTG GAG CAC AAC GGC ACA GAT GGC AAC AAC CGG AAC GAA GAC CGC CTG TGC CGC ACC 600

         610         620         630         640         650         660
GAG TTC GCT GTG CGC TCT GGG CTG CTC ACC GTC ATG GTG TGG GTG TCC AGG AAG GTC TTC   660
TTT CTA CCG GTC TTC TGC ACT GTG CTC AGT CTC ATC GTG TAC AGT CTC GGG AAC CAC TCG CGG 720
AGA CGC GGA GAT GCA GCG GGC GTG GCC TCG CTC GGC GAC CAG ACA GTG                   780
AAG ATG CTT Ggt gag tcc tgg cac ccg acc ttt ctt ccc cca gcc ctt ccc              840
cag cgg cct cta ttt ctc atc atc tcc gct ccc caa gtc tct caa gtc tct gtc          900
```

FIG.27A

```
        910         920         930         940         950         960

ttt ctc tgc ctc tct cac ctt ggt tct cgg tct cac tgc ttt tct tcc tgt ctt    960
ttc ctg tat ctt gtc cac gaa aaa gaa ccc tca tat tgg taa ttc ctt aaa acg agg aac   1020
ctt ggt ctg gga aaa ttg gtc caa gat gga aat acc tca cgg ttt att gag ccc cta att   1080
gtt aac ggt tta gct tct tgt ctc aca tag aat ttg tgg tta tca aag taa taa tat taa   1140
ggt aag cag gca ggt ttt aga aat ggg ttt aga aat cac tcc atg gta agt cta acc aca aat ttg   1200


        1210        1220        1230        1240        1250        1260

ggt cac tct gtt aag gac ggc tta tag atg tat ttt gtt tgt ttt caa tat tgg gat ttg   1260
ttt tct gcc ctg cat ctt gct ggg ctt cat att aca tcc act ctg ttt agt cta tgg ttt tgc   1320
cag gag ggg ctt cat ggt tcc ttc ttt ttc ttg tat ttg tct ccc cag taa   1380
tat agg cca gga tag ggt gga gaa gtc tca aac tgt cct tca gga agg tct   1440
ggg tac tga acg gtt act gca taa act ctg ctt ccc caa agg cat gtg ctt ggt gtg gta   1500


        1510        1520        1530        1540        1550        1560

aag tca aga tgg tgc tca tgt cca aga gga acc tct gat ctc act ttt caa ggg att   1560
tca tgt ttg ctg aca ttt aat act tgt tag ttt ttg cag ggg gat gat ttc tca ttt gca   1620
att tta tta ttc tca aat tct gca tgt cag aat gtt aga gat ttc tca ggg atg tca ggt   1680
tct gtt tcc aga tga gtg att gcc ctg tgt cct cca ttg gac tgt aaa ctc ata tgc acc   1740
aga cag ggt cta tgc tgc cgt ggt gca tag cct gca tcc atg tgt cac tta gtc cta aag   1800
```

FIG.27B

```
              1810      1820      1830      1840      1850      1860

aga agt tac taa taa cct act aat ctc act ggc atc tca atg ccg atc cca ttg   1860
tca tct gaa aat ttg aag ggg aca tta aag tgg cac agg gac cag aac aat att ttt ctc   1920
tca ttg ctg aat ttt aaa aac aat cta aaa aat tgg aat tct tga aga aac tat ctt ata   1980
tga cta aaa tga agc ctt ggg tgg gtg cta att att gtc tgg ctt acc tgc ccc ccc   2040
cac tac tta tat ctt tta gag atg aca cag act tgc ttt ccc tgt tac taa tcc caa   2100

              2110      2120      2130      2140      2150      2160

ttg cac att cag tcc ctt gat aga ctt act cta aaa atc taa gtt cag cgg tcc acg aaa   2160
cat aac aaa gcc tgt cct aaa aca gca aag aag aca aag gaa aga gca aga aag aaa   2220
gca aga aga gaa aag aga aca cag aag aag gtc ttt ccc cat tcc cta aca tac   2280
agg aat gga aat tat taa gtc tac gtg ata gcc aat gca tct gtt tct tca gta tgc cca   2340
caa ggg tgc tgc cgg cat tgc tca ggg ctg gag tat tta ctg gcc atg ctt gac ccc   2400

              2410      2420      2430      2440      2450      2460

agc atg gag ggt gag aag tgc tcc tgg gaa ctc tga tcc act gct gtg gag agc aaa   2460
cac ctg gcc tca ttt ata ctt gtt gtc tgt ata atg cat aat ggg gga taa tca tta   2520
cta aac tgt tta gct gct cct cat gtc agt cat caa tca caa agc aga gta att acc aca cag   2580
act ggg aag aag ctc agt gaa gat gat tgt tag cgg ttg gtc tga cag tct tgt gtg cta tag   2640
tgt tag acc caa cgg agg cag agg cag tat tta toa ggg cag ggt tcc atg ttt ccc gtg tta   2700
```

FIG.27C

```
                                        2760
                2740        2750         2760
aag agc aag aga tga tgt tca gta ggc atg cag ctc atg gtg aaa aga aag tcc aga   2760
ctt aaa gat gtg aag tga ttt gtg ctt tgc ccc acc ctg aca gtc tct ctc tgt gtg cst   2820
tca GCT GTG GTG TTT GCT TTC ATC CTC TGC TGG CTG CCC TTC CAC GTG GGA AGA TAC   2880
CTC TTT TCC AAG TCC TTC GAG CCT GGC TCT CTG GAG ATC GCT CAG ATC AGC CAG TAC TGC   2940
AAC CTG GTG TCC TTT GTC CTC TTC TAC CTC TTC GTC AGC GCT ATC AAC CCC ATT CTG TAC AAC   3000
                                        3060
            3040        3050         3060
ATC ATG TCC AAG AAG TAC CGG GTG GCA GTG TTC AAA CTG CTA GGA TTT GAA TCC TTC TCC   3060
CAG AGA AAG CTT TCC ACT CTG AAG GAT GAG AGT TCC CGG GCC TGG ACA AAG TCG AGC ATC   3120
AAC ACA TGA   3129
```

FIG.27D

```
          10        20        30        40        50        60
ATG TGG AAC GCG ACC CCC AGC GAG GAG CCT AAC GTC ACG TTG GAC CTG GAT TGG   60
GAC GCT TCC CCC GGC AAC GAC TCA CTG CCT GAC CTG CTG CCG CTG CCC GCT CCG  120
CTG CTG GCA GGC GTC ACC GCC ACC TGC GCG CTC TTC GTG GCG ATC TCA GGC AAC  180
CTG CTC ACT ATG CTG GTG GTG TCC TTC CGC GAG CTG CGC ACC AAC ACC CTC TAC  240
CTG TCC AGC ATG GCC TTC TCG GAT CTC ATC TTC CTG CTG ATG CCG GTG GAC CTC GTC  300

          310       320       330       340       350       360
CGC CTC TGG CAG TAC CCC TGG AAC TTC GGC GAC CTG CTC TGC AAA CTC TTC CAG TTT  360
GTC ACC GAG AGC TGC ACC TAC TGC CTC ACG GTC ACC ATC ACC GCG CTG AGC GTC CGC  420
TAC TTC GCC ATC TGC TTC CCT CTG CGG GCC AAG GTC GTC ACT AAG GGC CGC GTG AAG  480
CTG GTC ATC CTT GTC ATC TGG GCC GTG TCC GCT TTC GCG GGG CCC ATC TTC GTG CTG  540
GTG GGC GTG GAG CAC CAC GAA AAC GGC ACA GAT CCC CGG GAC AAC GAA TGC CGC GCC ACC  600

          610       620       630       640       650       660
GAG TTC GCT GTG CGC TCT GGG CTG CTC ACC GTC ATG GTG TGG TCC AGC GTC TTC TTC  660
TTT CTA CCG GTC TTC TGC TTC ACT GTG CTC TAC CTC ATC GGG AGG AAG CTA TGG CGG  720
AGA CGC GGA GAT GCA GCG GGC GTG TCG GGC GCC CTG CTC CGG GAC AAG CAC AAG GCA GTG  780
AAG ACG ATG CTT GCT GTG GTG TTT GCT GTG TGT TCC CTG TGC TGG GGA GTG CAC GTG GGA  840
AGA TAC CTC TTT TCC AAG TCC TTC GAG CCT GGC TCT CTG GAG ATC GCT CAG ATC AGC CAG  900
```

FIG.28A

```
                    910           920           930           940           950           960
TAC TGC AAC CTG GTG TCC TTT GTC CTC TTC TAC CTC AGC GCT GCC ATC AAC CCC ATT CTG   960
TAC AAC ATC ATG TCC AAG AAG TAC CGG GTG GCA GTG TTC AAA CTG CTA GGA TTT GAA TCC  1020
TTC TCC CAG AGA AAG CTT TCC ACT CTG AAG GAT GAG AGT TCC CGG GCC TGG ACA AAG TCG  1080
AGC ATC AAC ACA  1092
```

FIG.28B

```
         10        20        30        40        50

MWNATPSEEP EPNVTLDLDW DASPGNDSLP DELLPLFPAP LLAGVTATCV   50
ALFVVGISGN LLTNLVVSRF RELRTTTNLY LSSMAFSDLL IFLCMPLDLV  100
RLWQYRPWNF GDLLCKLFQF VSESCTYATV LTITALSVER YFAICFPLRA  150
KVVVTKGRVK LVILVIWAVA FCSAGPIFVL VGVEHENGTD PRDTNECRAT  200
EFAVRSGLLT VMVWVSSVFF FLPVFCLTVL YSLIGRKLWR RRGDAAVGAS  250


        260       270       280       290       300

LRDQNHKQTV KMLAVVVFAF ILCWLPFHVG RYLFSKSFEP GSLEIAQISQ  300
YCNLVSFVLF YLSAAINPIL YNIMSKKYRV AVFKLLGFES FSQRKLSTLK  350
DESSRAWTKS SINT 364
```
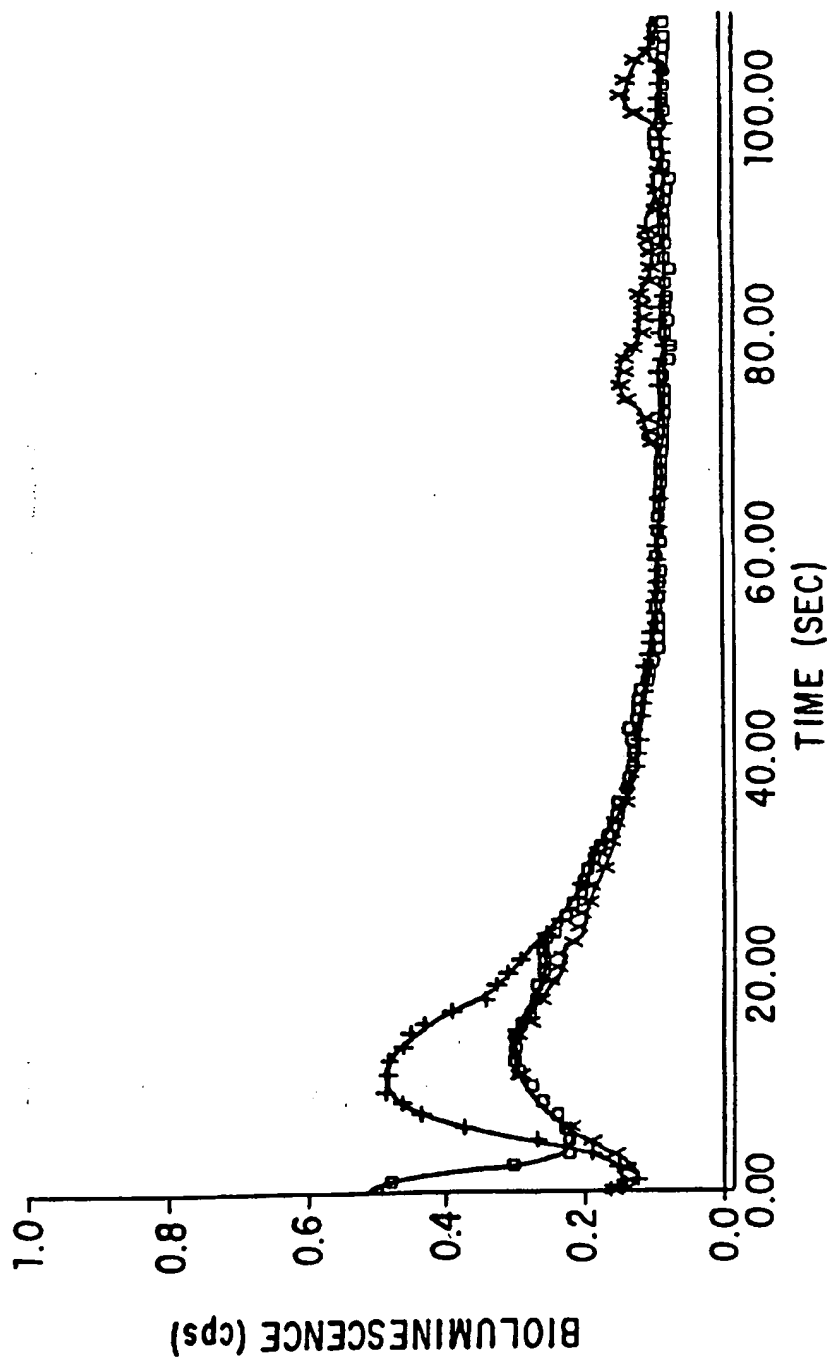
FIG.29

FIG. 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19442

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/566; C12N 15/87, 15/63; C07K 14/715; C12Q 1/02
US CL : 435/7.21, 29, 325, 320.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21, 325, 320.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, E	US 5,591,641 A (THORNER ET AL.) 07 January 1997, see column 4 line 5-63, column 19, line 10-25.	1, 3-14
A	SETHUMADHAVAN et al. Demonstration and characterization of the specific binding of growth hormone-releasing peptide to rat anterior pituitary and hypothalamic membranes. Biochem. Biophys. Res. Comm. 15 July 1991, Vol. 178, pages 31-37. See entire document.	1, 3-14
A, P	HOWARD et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 16 August 1996, Vol 273, pages 974-977.	1, 3-14

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 FEBRUARY 1997	0 4 MAR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MUKUL RANJAN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,410,513 A (MOMANY ET AL.) 18 October 1983, see entire document.	1, 3-14

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐   Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐   Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐   Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

    Please See Extra Sheet.

1. ☐   As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐   As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐   As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒   No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1, 3-14

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                          ☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAPUS, JICST-EPLUS, WPIDS
Search Terms : Growth Hormone Seretagogue Receptor, Growth Hormone releasing peptide, HRP, GHSR. Assay , G-protein receptor,

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1, 3-14, drawn to an assay to identify GHSR or GHSRR.
Group II, claim(s) 2, 15-18, drawn to an assay to identify growth hormone secretagogues.
Group III, claim(s) 19-22, drawn to a binding assay for ligand.
Group IV, claim(s) 23, drawn to ligands identified in assay of group II.
Group V, claim(s) 24, drawn to a binding assay for growth hormone secretagogue receptors.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I, II, III and V are to processes that either use materially different process steps or different products which are their special technical features. Pursuant to 37 CFR 1- 475(d), the ISA/US considers that unity of invention of invention does not exist between dissimilar methods which do not correspond to the main invention of the first group.

The ligands of Group IV is structurally and functionally different from, and does not share the special technical feature of the methods of groups I, III and V which are the process steps used by the methods.

Group IV does not share the special technical features of either of groups II or III because neither II nor II distinguish prior
art products meeting functional requirements of IV.